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FACULDADE DE MEDICINA
UNIVERSIDADE DO PORTO

HOW IMPORTANT IS THE URETHRA?

IMPACT OF SPINAL CORD INJURY ON THE URETHRA AND LOWER URINARY TRACT FUNCTION

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TESE DE DOUTORAMENTO APRESENTADA

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Em obediência ao disposto no Decreto-Lei 388/70, Artigo 8º, parágrafo 2, declaro que efetuei o planeamento e execução do trabalho experimental, observação do material e análise dos resultados, e redigi as publicações que fazem parte integrantes desta dissertação:

I. Ferreira A, Nascimento D, Cruz CD (2023), Molecular Mechanism Operating in Animal Models of Neurogenic Detrusor Overactivity: A Systematic Review Focusing on Bladder Dysfunction of Neurogenic Origin. International journal of Molecular Sciences 24:3273.

II. Ferreira A, Chambel SS, Avelino A, Nascimento D, Silva N, Cruz CD (2024) Urinary dysfunction after spinal cord injury: Comparing outcomes after thoracic spinal transection and contusion in the rat. Neuroscience, 557: p. 100-115.

III. Ferreira A, Chambel SS, Avelino A, Cruz CD (2022) Spinal Cord Injury Causes Marked Tissue Rearrangement in the Urethra—Experimental Study in the Rat. International Journal of Molecular Sciences, 23(24): p. 15951.

IV. Ferreira A, Chambel SS, Reguenga C, Avelino A, Cruz CD (2025) Development of urinary impairment after Spinal Cord Injury reflects altered urethral serotonin signalling: an experimental study in female mice- Manuscript accepted for publication in *Scientific Reports.*

À Professora Doutora Célia Duarte Cruz

Aos meus Pais

Prólogo

"Nothing in life is to be feared, it is only to be understood.
Now is time to understand more, so that we may fear less". Marie Curie

A conclusão deste doutoramento é, sem dúvida, um dos marcos mais gratificantes da minha existência. Entre sacrifícios e obstáculos que, em muitos momentos quase me derrubaram (literalmente!), aprendi que a resiliência se constrói nos dias difíceis, que o conhecimento se aprofunda nas perguntas sem resposta, e que a persistência nem sempre é uma escolha. Termino esta caminhada com sentimento de dever cumprido, e com a convicção de que embora árduo, o caminho foi recompensador. O sucesso desta conquista deve-se, em grande parte, às várias pessoas que foram amparando o meu caminho, e a elas presto, de seguida, os meus agradecimentos.

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List of abbreviations

5-HT - Serotonin

Ach - Acetylcholine

ATP - Adenosine Triphosphate

BoNT/A - Botulinum toxin A

CGRP - Calcitocin gene-related peptide

CNS - Central Nervous System

DRG - Dorsal Root Ganglia

DSD- Detrusor Sphincter Dyssynergia

EUS - External Urethral Sphincter

GAP43 - Growth Associated Protein 43

IUS - Internal Urethral Sphincter

LUT - Lower urinary tract

NA - Noradrenaline

NDO - Neurogenic Detrusor Overactivity

NGF - Nerve Growth Factor

NO - Nitric Oxide

PAG - Periaqueductal Gray

PMC - Pontine Micturition Centre

PSC - Pontine Storage Centre

SCC - Spinal Cord Contusion

SCI - Spinal Cord Injury

SCT - Spinal Cord Transection

TH - Tyrosine Hydroxylase

TPH1 - Tryptophan Hydroxylase 1

TRPV1 - Transient Receptor Potential 1

VAChT - Vesicular Transporter of Acetylcholine

WT - Wild Type

Abstract

Micturition, defined as the storage and periodic elimination of urine, is dependent upon the synchronised activity of the bladder, the urine reservoir, and urethral sphincters, which control the bladder neck opening for urine expulsion through the urethra. Such coordination relies on complex neuronal networks that integrate afferent and efferent inputs travelling between the lower urinary tract (LUT), peripheral neurons and supraspinal centres, conveyed by the spinal cord. Injuries to this pathway can lead to urinary impairment, the severity of which reflects the level and severity of the neuronal damage. Neurogenic Detrusor Overactivity (NDO) is one of the most common manifestations of LUT dysfunction following central neurologic injury. Animal models have been essential for understanding their pathophysiological mechanisms and have helped to develop therapeutic strategies focusing on managing symptoms and improving patients' quality of life.

Spinal cord injury (SCI) is one of the most extensively studied causes of NDO, which develops as a result of the interruption of ascending and descending neuronal tracts regulating micturition. Following an initial period of little or no bladder reflex activity, neuroplastic events within the lumbosacral spinal cord mediate the development of an automatic micturition reflex, restricted to the spinal cord and functioning in the absence of brain input. Therefore, voluntary control over voiding is lost, and micturition becomes largely inefficient due to the simultaneous presence of strong and involuntary bladder contractions (NDO) and impaired detrusor-urethral sphincter coordination (Detrusor-sphincter dyssynergia- DSD), ultimately leading to urinary incontinence and other urinary complications.

Animal models used in SCI-induced urinary dysfunction research have largely relied on complete transection of the spinal cord due to their technical simplicity and higher reproducibility rates. However, these offer limited translational relevance compared to contusion or compression models, which more accurately replicate human SCIs. Also, for decades, the treatment for SCI-induced urinary impairments focused essentially on bladder alterations, overlooking the urethra´s potential role in LUT dysfunction. Emerging evidence, however, suggests that the urethra significantly contributes to efficient bladder emptying, raising the question of whether SCI-induced urinary impairments could also reflect changes in urethral function.

Publication I systematically reviewed the different methods to study NDO in animal models of disease, aiming to elucidate its underlying molecular mechanisms. Among the analysed studies, SCI was the most commonly used approach, due to its technical ease and reliable functional outcomes. Rodents, preferentially rats, were the most used organisms, with females often being preferred due to the absence of the prostate that facilitates bladder manipulation. The consequences of SCI on urinary function were primarily assessed by urodynamic testing via cystometry. Following SCI, the bladder undergoes upregulation of inflammatory, apoptotic, ischemia-related, and fibrosis-associated proteins. Moreover, neuronal signalling is disrupted, as indicated by reduced expression of purinergic, cholinergic, and adrenergic receptors, along with a general decline in neuronal marker expression. Within neuronal tissue, NDO coursed with high levels of neurotrophic factors, apoptosis-associated molecules, and ischemia-related markers. Alterations in the urethra were rarely assessed, reflecting a knowledge gap about the role of possible SCI-induced urethral changes in post-SCI urinary dysfunction.

Ferreira A, Nascimento D, Cruz CD (2023), Molecular Mechanism Operating in Animal Models of Neurogenic Detrusor Overactivity: A Systematic Review Focusing on Bladder Dysfunction of Neurogenic Origin. International journal of Molecular Sciences 24:3273.

Publication II investigated differences in LUT function and innervation between distinct SCI models. Female rats were submitted to spinal cord transection (SCT) or spinal cord contusion (SCC) of varying severities at the thoracic level, the latter using a cost-effective, custom-built contusion device developed by our team. Severely contused rats displayed prolonged spinal shock periods and a tendency toward increased post-void residual volumes, with intermediate effects observed in complete transectioned animals. The minor impact was seen in animals submitted to mild contusion, but cystometric evaluation failed to reveal significant changes in urodynamic parameters between the investigated SCI models. Immunohistochemistry at the lumbosacral spinal cord revealed sprouting of sensory neurons and loss of cholinergic fibres after SCI, which correlated with injury severity and, therefore, were most pronounced in SCT animals. Noradrenergic fibres were also affected, irrespective of the SCI model. At the LUT level, sensory, cholinergic and noradrenergic denervation was present, irrespective of the injury method. These findings suggest that although transection and contusion injuries lead to similar alterations in LUT function and innervation, they likely arise through distinct neuroplastic mechanisms at the lumbosacral spinal cord level.

Ferreira A, Chambel SS, Avelino A, Nascimento D, Silva N, Cruz CD (2024) Urinary dysfunction after spinal cord injury: Comparing outcomes after thoracic spinal transection and contusion in the rat. Neuroscience, 2024. 557: p. 100-115.

Publication III described the consequences of SCI on the urethra, utilising the model of complete thoracic spinal cord transection in female rats. Urodynamic recordings revealed time-dependent alterations in post-SCI bladder function, with signs

of bladder areflexia at one week post-injury that were replaced by NDO indicators at four weeks post-injury. This coursed with significant alterations in urethral morphology, including time-dependent epithelium reorganisation, atrophy of the smooth muscle of the internal urethral sphincter (IUS), and fibrosis of the striated muscles forming the external urethral sphincter (EUS). Innervation was also compromised, with signs of denervation in sensory and sympathetic urethral nerve fibres. These findings highlighted the impact of SCI on the rat urethra and raised questions about whether changes observed could affect urethral function and might contribute to SCI-induced urinary dysfunction.

Ferreira A, Chambel SS, Avelino A, Cruz CD (2022) Spinal Cord Injury Causes Marked Tissue Rearrangement in the Urethra—Experimental Study in the Rat. International Journal of Molecular Sciences, 23(24): p. 15951.

Publication IV investigated the consequences of SCI on the urethro-vesical neuronal crosstalk mediated by peripheral serotonin (5-HT), produced by paraneuronal cells lining the urethra. Experiments were performed in Wild-Type (WT) and Tryptophan hydroxylase 1 null (*tph1*$^{-/-}$) female mice, which lack peripheral 5-HT production, and submitted to complete spinal cord transection at T8/T9 level. Data demonstrated increased expression of urethral 5-HT^{+} paraneuronal cells in response to SCI 4 weeks post-injury, with bladder hyperactivity in WT mice. In *tph1*$^{-/-}$ injured mice, 5-HT^{+} cells were absent in the urethra, and cystometries evidenced a less affected bladder function in comparison with WT animals. Urethral smooth muscle atrophy was evident 4 weeks post-injury in both genotypes, but striated muscle fibrosis was only detected in *tph1*$^{-/-}$ 4w SCI mice. The expression of sensory and cholinergic markers was increased only in the presence of peripheral 5-HT (i.e. in WT mice), suggesting that 5-HT depletion might block expansion of sensory and cholinergic innervation in the IUS after SCI. Additional

experiments blocked 5-HT signalling in WT SCI mice using selective 5-HT_2 and 5-HT_3 receptor antagonists, administered for 28 days. Our results indicated that even though the emergence of NDO was not prevented, beneficial changes in bladder function occurred in animals receiving the selective 5-HT_2 antagonist. This study expands on the work described in *Publication III* and further demonstrates the impact of SCI on urethral function.

Ferreira A, Chambel SS, Avelino A, Cruz CD (2025) Development of urinary impairment after Spinal Cord Injury reflects altered urethral serotonin signalling: an experimental study in female mice. Manuscript accepted for publication in Scientific Reports.

Resumo

A micção, definida como o conjunto de processos que envolvem o armazenamento e a eliminação periódica da urina, é dependente da atividade sincronizada da bexiga, onde a urina é armazenada, e dos esfíncteres da uretra, que controlam a abertura do colo da bexiga para a expulsão de urina através da uretra até ao exterior. Esta coordenação está dependente de uma complexa rede neuronal, que integra sinais nervosos entre órgãos do trato urinário inferior (bexiga e uretra), neurónios periféricos, e vários centros supraespinhais, sendo estes conduzidos através da medula espinhal. Qualquer dano ocorrido neste percurso pode resultar em disfunção urinária, cuja gravidade depende do nível e da extensão do dano neuronal. A atividade neurogénica do detrusor (do inglês, *Neurogenic Detrusor Overactivity- NDO*) é uma das principais manifestações de disfunção urinária recorrente de lesões no sistema nervoso central. O uso de modelos animais tem-se mostrado essencial para a compreensão dos mecanismos patofisiológicos da doença, e consequentemente, para o desenvolvimento de terapias focadas na redução dos sintomas e na melhoria da qualidade de vida dos doentes.

As lesões vertebro-medulares são uma das causas mais estudadas da atividade neurogénica do detrusor, que decorre da interrupção abrupta dos circuitos neuronais responsáveis pelo controlo da micção. Após um período inicial de atividade vesical reduzida, ou mesmo ausente (choque medular), vários eventos neuroplásticos ao nível da medula lumbosagrada, promovem o estabelecimento de um reflexo miccional alternativo restrito á medula espinhal, que funciona na ausência de controlo supramedular. Consequentemente, a micção deixa de estar sobre o controlo voluntário do indivíduo e torna-se automática, todavia amplamente ineficaz, devido à ocorrência

de contrações vesicais intensas e involuntárias (NDO) e à dissinergia entre a contração do detrusor e dos esfíncter uretrais (do ingles, *Detrusor Sphincter Dyssinergia- DSD*), que resultam, entre outras complicações, em incontinência urinária.

Os modelos animais usados na investigação da disfunção urinária recorrente de lesões medulares, têm-se baseado, em grande parte, na transeção completa da medula espinhal, uma técnica simples e altamente reprodutível. No entanto, estes modelos apresentam uma relevância translacional limitada quando comparados com outro tipo de modelos, como é o caso da contusão ou da compressão medular, que reproduzem de forma mais fidedigna as lesões observadas em humanos. Além disso, durante décadas, o tratamento da disfunção urinária baseou-se essencialmente nas consequências da lesão na bexiga, descuidando o possível papel funcional da uretra nos mecanismos patofisiológicos da doença. Contudo, estudos recentes indicam que a uretra desempenha um papel importante no esvaziamento vesical, levantando a hipótese de que a disfunção urinária recorrente de lesão medular possa também refletir alterações no funcionamento da uretra.

A ***Publicação I*** reviu sistematicamente os diferentes métodos usados no estudo da atividade neurogénica do detrusor em modelos animais, tendo como objetivo elucidar os seus mecanismos moleculares subjacentes. De entre os estudos analisados, os modelos de lesão medular destacaram-se como a técnica mais usada, em virtude da sua facilidade técnica e da possibilidade da obtenção de resultados funcionais consistentes e facilmente reprodutíveis. Roedores, preferencialmente ratazanas, foram o organismo mais utilizado, sendo as fêmeas o sexo preferencial, devido á inexistência da próstata que facilita a manipulação vesical. As consequências das lesões na atividade vesical foram avaliadas essencialmente por avaliação urodinâmica via cistometria. Após

lesão medular, observam-se na bexiga aumentos na expressão de proteínas inflamatórias, apoptóticas, isquémicas e fibroticas. Existe também comprometimento da sinalização neuronal, evidenciado pela redução na expressão de receptores purinérgicos, colinérgicos e adrenérgicos, bem como por uma diminuição generalizada na expressão de marcadores neuronais. No tecido nervoso, observam-se níveis elevados de fatores neurotróficos, de moléculas relacionadas à apoptose, e marcadores de isquemia. As alterações na uretra foram parcamente investigadas, evidenciando uma lacuna no conhecimento sobre o possível papel das modificações uretrais na disfunção urinária após lesão medular.

Ferreira A, Nascimento D, Cruz CD (2023), Molecular Mechanism Operating in Animal Models of Neurogenic Detrusor Overactivity: A Systematic Review Focusing on Bladder Dysfunction of Neurogenic Origin. International journal of Molecular Sciences 24:3273.

A ***Publicação II*** investigou as diferenças na função urinária, bem como nos padrões de enervação nos órgãos do trato urinário baixo e medula lumbosagrada, entre diferentes modelos de lesão medular. Neste estudo foram usados ratazanas fêmeas, submetidas a transeção medular completa ou contusão medular de diferentes intensidades ao nível T8/T9, sendo esta última obtida através de um dispositivo de contusão simples e económico desenhado pela nossa equipa. Animais submetidos a contusão severa apresentaram períodos prolongados de choque medular, com uma tendência para maiores volumes de retenção urinária por períodos mais prolongados. Efeitos intermédios foram observados nos animais submetidos a transeção completa, sendo os submetidos a contusão moderada os que apresentaram valores de retenção mais baixos. No entanto, a avaliação urodinâmica por cistometria não revelou alterações significativas no que toca á função vesical entre os modelos estudados. A avaliação

imunohistoquímica de vários marcadores neuronais ao nível da medula lumbosagrada revelou crescimento neuronal de fibras sensitiva e perda de fibras colinérgicas após lesão medular, correlacionados com a severidade da desta e, portanto, mais proeminentes em animais submetidos a transeção medular completa. Foi também detetada a perda de fibras noradrenégicas, mas independentemente do modelo de lesão. Ao nível da bexiga e uretra, foi observada desenervação nas fibras sensitivas, colinérgicas e noradrenegicas, independentemente do modelo de lesão usado. Estes dados sugerem que embora os modelos de transeção e contusão resultem em consequências semelhantes ao nível da função urinária e enervação da bexiga e uretra, estas mudanças possivelmente decorrem de mecanismos neuroplásticos distintos ao nível da medula lumbosagrada.

Ferreira A, Chambel SS, Avelino A, Nascimento D, Silva N, Cruz CD (2024) Urinary dysfunction after spinal cord injury: Comparing outcomes after thoracic spinal transection and contusion in the rat. Neuroscience, 2024. 557: p. 100-115.

A ***Publicação III*** focou-se na descrição das consequências da lesão medular sobre a uretra, com recurso ao modelo de transeção medular completa em ratazanas fêmeas ao nível torácico. A análise urodinâmica revelou alterações temporais na função vesical após lesão, com sinais de areflexia vesical uma semana após lesão, e sinais de hiperatividade neurogénica do detrusor quatro semanas após a lesão. Estas ocorreram paralelamente com alterações morfológicas na uretra, incluindo reorganização do epitélio, atrofia do músculo liso do esfíncter uretral interno, e fibrose da musculatura estriada do esfíncter uretral externo. A inervação da uretra foi também significativamente afetada, com evidência de denervação das fibras sensitivas e simpáticas. Estes dados destacaram o impacto das lesões medulares na estrutura da

uretra e levantaram questões acerca da possibilidade de estas afetarem a sua função, e potencialmente contribuírem para a disfunção urinária provocada por lesão medular.

Ferreira A, Chambel SS, Avelino A, Cruz CD (2022) Spinal Cord Injury Causes Marked Tissue Rearrangement in the Urethra—Experimental Study in the Rat. International Journal of Molecular Sciences, 23(24): p. 15951.

A ***Publicação IV*** investigou as consequências das lesões medulares na comunicação neuronal entre a bexiga e a uretra mediata pela serotonina (5-HT), produzida na uretra por células epiteliais especializadas chamadas de paraneurónios. Os estudos foram conduzidos em murganhos fêmea *Wild-Type (WT)* e *knockout* para o gene *tph1, q*ue codifica para a produção periférica de 5-HT *(tph1*$^{-/-}$), e submetidos a transeção medular completa ao nível T8/T9. Os dados indicaram um aumento da expressão de paraneurónios serotonergicos na mucosa da uretra em resposta á lesão, nomeadamente após 4 semanas, acompanhado de sinais de hiperatividade vesical nos animais WT. Nos animais tph1$^{-/-}$, não foram detetadas células serotonergicas na uretra, e a avaliação urodinâmica evidenciou um menor comprometimento da função vesical em comparação com os animais WT. A atrofia do musculo liso do esfíncter interno foi evidente em ambos os genótipos quatro semanas após lesão, todavia, a fibrose da musculatura estriada do esfíncter externo foi observada apenas nos animais *tph1*$^{-/-}$. A expressão de marcadores de neurónios sensitivos e colinérgicos aumentou apenas nos animais WT, sugerindo que a depleção da 5-HT periférica pode impedir a expansão destes neurónios uretrais após lesão medular. Experiências adicionais com animais lesionados WT bloquearam a sinalização serotonérgica com recurso á administração de antagonistas seletivos dos receptores 5-HT_2 e 5-HT_3 durante 28 dias. Embora nenhum dos tratamentos tenha conseguido prevenir a emergência da hiperatividade do detrusor, foram detetadas

alterações benéficas na atividade vesical nos animais tratados com o antagonista dos recetores 5-HT_2. Este estudo expande os resultados descritos na publicação anterior, demonstrando mais uma vez, o impacto das lesões medulares na função da uretra.

Ferreira A, Chambel SS, Avelino A, Cruz CD (2025) Development of urinary impairment after Spinal Cord Injury reflects altered urethral serotonin signalling: an experimental study in female mice. Manuscript accepted for publication in Scientific Reports.

State of the art

This section was partially based on the following publications:

Ferreira A, Nascimento D, Cruz CD (2023): Molecular Mechanism Operating in Animal Models of Neurogenic Detrusor Overactivity: A Systematic Review. International Journal of Molecular Sciences. doi: 10.3390/ijms2 (***Publication I***)

Ferreira A, Cruz CD (2021) The urethra in continence and sensation: Neural aspects of urethral function. Neurourology and Urodynamics. doi: 10.1002/nau.24632 (***Past publication***)

1. The neural control of micturition

Micturition is the process comprising the storage and periodic elimination of urine. Correct micturition behaviours depend on the synchronised activity of two functional units of the lower urinary tract (LUT). The first one is the bladder, the urine reservoir, and the second one is the bladder outlet, composed of the bladder neck, the urethra, and the urethral sphincters, which control expulsive urine flux (Yoshimura, 1997; Fowler et al., 2008). LUT function is under voluntary and learned control, developed during maturation of the central nervous system (CNS) (Fowler et al., 2008). Immediately after birth, voiding reflexes are primitive and presumably regulated by non-neural mechanisms. During early childhood, micturition evolves into a reflex mechanism dependent on neuronal pathways solely present in the spinal cord, being controlled by supraspinal centres only later in life, as the CNS completely matures (de Groat, 2002; Fowler et al., 2008).

Neuronal control of micturition is dependent on four basic mechanisms that rely on peripheral and central nerves. The primary *afferent neurons* (1) carry information about bladder filling and urethral flow to the *spinal interneurons* (2) present in the lumbosacral (L5-S1) spinal cord. These interneurons send this information to higher *supraspinal centres* (3), where the afferent information is integrated and a decision to void is made. The *efferent neurons* (4) carry the efferent input back to LUT via descending projections, where the motor response will be reproduced (Andersson and Gratzke, 2008) (Figure 1).

1.1. Peripheral innervation: afferent and efferent neurons

Afferent innervation

Afferent neurons, also known as sensory neurons, are responsible for the transport of the sensory information generated in LUT organs to the CNS. Their processes innervate

the LUT and run in the pelvic and hypogastric nerves (if originating in the bladder), or in the pudendal and hypogastric nerves (if coming from the urethra) (de Groat et al., 2015). Afferent neurons are pseudounipolar. Their cell bodies are located in the lumbosacral dorsal root ganglia (DRG), at the L5-S1 levels, and project to the spinal cord, where they establish synapses with interneurons involved in spinal guardian reflexes or with projection neurons connected to high supraspinal regions (Fowler et al., 2008; de Groat et al., 2015) (Figure 1). Afferent neurons innervating the LUT are glutamatergic (Morgan et al., 1981; De Biasi and Rustioni, 1988) and can be grouped into Aδ and C-fibres. Lightly myelinated Aδ fibres respond to passive distention of the bladder wall during filling and promote detrusor contraction (de Groat et al., 2015). In contrast, C-fibres, which are small unmyelinated fibres, are generally insensitive to bladder distention in physiological conditions. C-fibres are only activated in cases of high-intensity stimuli, which generate noxious sensory input, or by innocuous stimuli in the presence of inflammation (Andersson and Gratzke, 2008; de Groat et al., 2015). In pathological conditions involving inflammatory components, C-fibres lower their activation threshold and initiate a cascade of events that lead to peripheral sensitization, a mechanism central to several LUT dysfunction disorders (Grundy et al., 2019).

Efferent innervation

The efferent control of the LUT is mediated by autonomic (sympathetic and parasympathetic) and somatic motor neurons (Fowler et al., 2008; de Groat et al., 2015) (Figure 1). The parasympathetic preganglionic neurons originate in the sacral spinal cord segments (S2-S4) and run their processes in the pelvic nerve. They synapse with postganglionic neurons located in the pelvic plexus (in the rat) or the intramural ganglion

within the bladder wall (in the human and guinea pig). The parasympathetic nerves provide excitatory action in the bladder's smooth muscle. This occurs via Acetylcholine (ACh) acting on postjunctional muscarinic receptors, or Adenosine Triphosphate (ATP) acting on purinergic receptors, which results in detrusor contraction during voiding (Fowler et al., 2008; de Groat et al., 2015). In the urethra, the parasympathetic innervation has an inhibitory function, inducing the relaxation of the smooth muscle of the urethral sphincter via nitric oxide (NO) (Bennett et al., 1995; Andersson and Gratzke, 2008; Fowler et al., 2008).

The sympathetic preganglionic neurons originate from the intermediolateral column of the thoracolumbar spinal cord (T12-L2) and synapse with postganglionic neurons in the inferior mesenteric ganglia, running in the hypogastric nerve and the inferior mesenteric plexus (Fowler et al., 2008). Sympathetic neurons function via noradrenaline (NA) acting on β3 adrenergic receptors in the bladder smooth muscle (Andersson and Wein, 2004; Andersson and Gratzke, 2008; Fowler et al., 2008), inducing its relaxation during storage. However, the function of sympathetic neurons is thought to be more important in the bladder neck and urethra. In this case, sympathetic axons run through the paravertebral chain and enter the pelvic chain, acting on α1 adrenergic receptors. This induces urethral smooth muscle constriction and the closing of the bladder neck during storage, preventing urine leakage (Fowler et al., 2008; de Groat et al., 2015).

Somatic neuronal fibres arise from motor neurons of Onuf´s nucleus, located in the lamina IX of the ventral horn of the sacral spinal cord (de Groat et al., 2015). Somatic neurons run in the pudendal nerve and establish contact with muscle fibres of the striated muscle of the urethral sphincter. They function by releasing Ach acting on

nicotine receptors, inducing external urethral sphincter contraction during storage (C. de Groat, 2001; de Groat, 2006; Fowler et al., 2008).

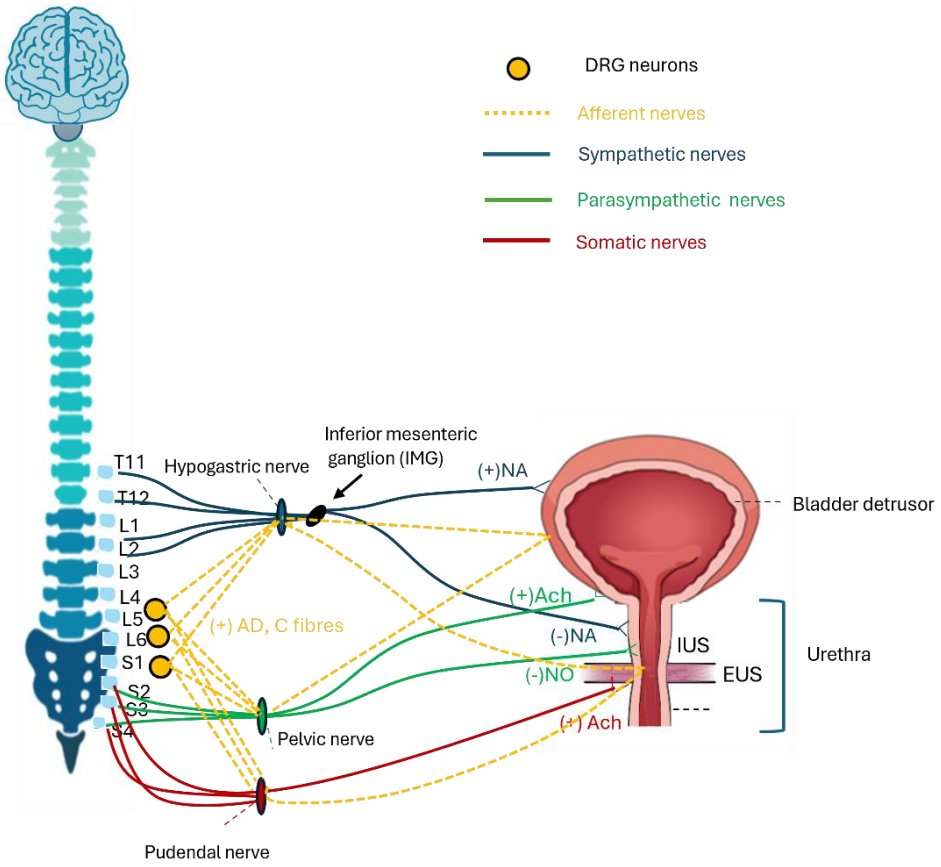


Figure 1. Afferent and Efferent innervation of the female lower urinary tract. In yellow, sensory fibres (ADelta or c-fibres) originated in the bladder run in the hypogastric and pelvic nerves, and in the pelvic and pudendal nerve if originated from the urethra. Their cell bodies are located in the lumbosacral dorsal root ganglia (DRG) and project to the superficial laminae of the L6-S1 spinal cord segments. In blue, sympathetic nerves from the T11-L2 spinal cord segments run through the inferior mesenteric ganglion and the hypogastric nerve. They use noradrenaline (NA) as an excitatory mediator acting both on the bladder and urethra. In green, parasympathetic preganglionic fibres arise from S2-S4 spinal cord segments and run through sacral roots and pelvic nerve. In the bladder, the excitatory effect of parasympathetic fibres is mediated by Acetylcholine (ACh) and Adenosine Triphosphate (ATP), whereas in the urethra it has an inhibitory function trough the Nitric oxide (NO). In red, somatic motor nerves arise from the S2-S4 motor neurons and run through the pudendal nerve. Adapted from Ferreira et al., 2022.

1.2. Central pathways: spinal cord and supraspinal neurons

Spinal cord neurons

Neurons involved in afferent pathways project to spinal cord regions and establish synaptic contact with interneurons and projection neurons. Interneurons are engaged in the maintenance of spinal reflexes during storage, making local connections in the spinal cord that can be inhibitory or excitatory. In turn, projection neurons send their long processes to high supraspinal areas via the spinothalamic tract that supplies the spinal-bulbo-spinal reflex responsible for voiding. Projection neurons are located in the dorsal commissure, the superficial dorsal horn and the parasympathetic nucleus (Fowler et al., 2008; de Groat et al., 2015).

Efferent control over the LUT involves parasympathetic, sympathetic and somatic mechanisms. Parasympathetic and sympathetic preganglionic neurons are located in the intermediate grey matter of spinal sacral and lumbar spinal segments, respectively. These neurons project into the dorsal commissure, the lateral funiculus, and the lateral dorsal horn of the spinal cord. The distribution of sympathetic dendrites is currently not fully understood (Fowler et al., 2008). Somatic motor neurons innervating the external urethral sphincter are located in the ventral horn (lamina IX) in Onuf ’s nucleus (de Groat, 2006; Fowler et al., 2008; de Groat et al., 2015).

Supraspinal centres

The supraspinal control of micturition depends on multiple neuronal populations spread across several regions (Fowler et al., 2008), including in the periaqueductal grey matter (PAG), the pontine micturition centre (PMC), and in particular cell groups of the caudal and preoptic hypothalamus, as well as in the cerebral cortex (particularly in the medial frontal cortex). Non-specific regions include the raphe nuclei, the locus coeruleus

and nuclei in the brain stem, which harbour non-specific 'level-setting' mechanisms with diffuse spinal projections (Holstege, 2005; de Groat, 2006; Fowler et al., 2008; de Groat et al., 2015; Keller et al., 2018). All these brain regions share interconnections and project to the lumbosacral spinal cord regions, where they coordinate the activity of the bladder, urethra, and urethral sphincter to ensure proper micturition.

1.3. Storage and voiding reflexes

The neuronal pathways controlling micturition are organised as a simple on-off switch that maintains a reciprocal relationship between the bladder's contraction and the urethral sphincters. When storage reflexes are activated, voiding mechanisms are inhibited, and vice versa. This perfect synchronisation is the key to proper micturition in healthy individuals (Fowler et al., 2008).

Storage reflexes

During storage, as the bladder fills, mechanoreceptors in the bladder generate a low-level sensory firing that reaches the L5-S1 spinal cord segments through sensory neuronal tracts. This results in maintenance of the spinal guardian reflex, which encompasses activation of the sympathetic neurons innervating the bladder neck and urethra, resulting in urethral sphincter contraction. At the same time, parasympathetic circuits in the bladder are silent, enabling the quiescence of the detrusor and urinary continence (Vilensky et al., 2004; Andersson and Gratzke, 2008; Fowler et al., 2008; Drake et al., 2010). Although this process is mainly controlled by spinal cord neurons, the involvement of a set of supraspinal neurons located in the dorsolateral pons is also acknowledged (Pontine Storage Centre - PSC). Descending input from this region activates somatic neurons that innervate the external urethral sphincter, increasing

urethral sphincter resistance and preventing urine leakage (Drake et al., 2010) (Figure 2A).

Voiding reflexes

As intravesical pressure reaches a certain threshold (designated as micturition threshold, which varies across individuals), bladder afferents generate a sudden and strong afferent input, which is conveyed to the spinal cord and activates the spinobulbospinal reflex pathway (Fowler et al., 2008). As this reflex is set in motion, afferent information travels through the spinal cord, reaching the PMC and then is conveyed to higher supraspinal structures, where the sensation of bladder fullness is integrated (Keller et al., 2018; Verstegen et al., 2019). If the individual decides to void, this information is relayed from the cortex back to the PMC, which will activate its descending projections. This results in inhibition of the sympathetic and somatic input into the bladder neck and external urethral sphincter, along with activation of the parasympathetic inhibitory tone by NO, resulting in urethral sphincter relaxation. In quick succession, parasympathetic neurons at the spinal cord generate a strong cholinergic outflow to the bladder, inducing detrusor contraction and urine release (Yoshimura, 1997; C. de Groat, 2001; Fowler et al., 2008; Drake et al., 2010; Keller et al., 2018; Verstegen et al., 2019) (Figure 2B).

The switching between storage and voiding responses is a voluntary learned behaviour, and this decision is made considering the social appropriateness of urinating. The PAG plays an important role in voiding control, which is dependent on the conscious registration of bladder filling, but also on the extent to which it is acceptable to delay voiding if urination is not convenient at the moment. In addition to receiving afferent information from the LUT and sending this information to higher brain centres, the PAG

serves as a relay station, also receiving input from supraspinal structures, such as the prefrontal cortex or the hypothalamus (Tish and Geerling, 2020). These regions can inhibit the excitatory input to the PMC and thus delay urination by maintaining the spinal guardian reflex even in the presence of a full bladder. When the occasion is acceptable and there is an appropriate place to void, the prefrontal cortex interrupts the tonic suspension of the PAG input to PMC, and voiding occurs (Fowler et al., 2008; Drake et al., 2010).

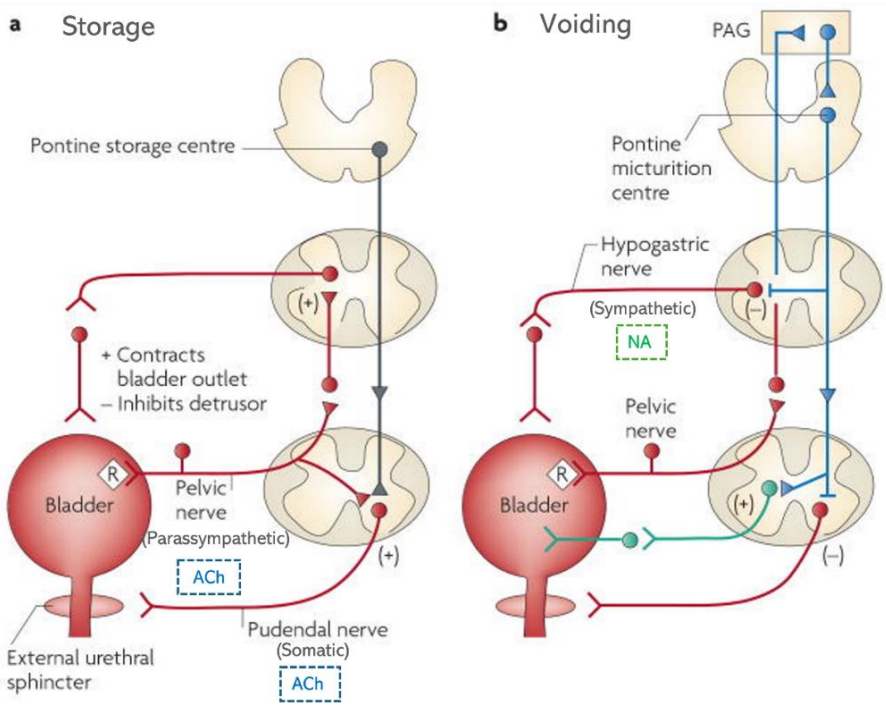


Figure 2. Neuronal pathways implicated in storage and voiding. (**A**) Urine storage: sympathetic and autonomic outflow through the hypogastric and pudendal nerves closes the bladder neck and contracts the urethral sphincter to urine storage; (**B**) Voiding: The inhibition of sympathetic and somatic urethral stimulation provokes sphincter relaxation, at the same time that parasympathetic stimulation in the bladder by pelvic nerves induces the contraction of the detrusor, enabling urine to flow. Sympathetic efferent and sensory afferent innervation from the spinal cord to the bladder and urethra is depicted in red. Spinobulbospinal reflex pathways are represented in blue. Parasympathetic outflow to the bladder is mentioned in green. Adapted from Fowler et al, 2006.

2. The role of the urethra in the neuronal control of micturition

For decades, the urethra was considered a simple, passive fibromuscular tube whose function was limited to urine expulsion. However, Humans can feel urethral distention, the insertion of a catheter or warm fluid into the urethra, and noxious stimulation (Wyndaele, 1998; Shafik et al., 2003; Birder et al., 2014). This means that the urethra is equipped with machinery capable of sensing, transmitting, and generating neuronal signals. Over the years, several studies have highlighted that the urethra is not a passive bystander but actively participates in micturition control, where the urethral sensory mechanisms play a key role (Birder et al., 2014; Eggermont et al., 2019; Ferreira and Duarte Cruz, 2021).

2.1. Anatomy, histology, and innervation of the urethra

The urethra is a small fibromuscular tube that connects the bladder at its upper end to the exterior of the body at its lower end, allowing urine transport and elimination. In males, the urethra extends along the entire length of the penis, passing through the centre of the prostate, and is divided into three sections: prostatic, membranous, and spongy urethra. Besides serving as a channel for urine expulsion, the urethra is also an important component of the male reproductive system, functioning as an extragenital duct for semen. In females, the urethra is shorter and used exclusively for urination. The female urethra begins in the bladder neck and embeds in the anterior wall of the vagina, inclining through a gap in the pelvic floor and terminating in the external zone between the clitoris and the vaginal opening (Pradidarcheep et al., 2011; Ferreira and Duarte Cruz, 2021).

The urethral wall comprises four essential components: the epithelium (1), a lamina propria (2) containing connective tissue and microvasculature, and layers of smooth (3) and striated muscle (4) that in its proximal portion are arranged to form the urethral sphincter (Ferreira and Duarte Cruz, 2021; McCloskey et al., 2024) (Figure 4).

Urethral mucosa: Epithelium and lamina propria

In the LUT, the mucosa is composed of a specialised epithelium, the urolothelium, and its supporting lamina propria. The urothelium has important sensory and signalling properties that mediate complex communication between the urinary space and the underlying lamina propria and muscularis (Fowler et al., 2008; Birder et al., 2010; Birder and Andersson, 2013). In the bladder, the urothelium is composed of at least three layers of epithelial cells forming a transitional epithelium and assuring an important barrier function. In the proximal urethra, the urothelium presents fewer layers and becomes a stratified or pseudo-stratified columnar epithelium at the urethral meatus (Pradidarcheep et al., 2011) and lacks the specialised differentiation markers characteristic of urothelial cells (Figure 3) (Sun, 2006; Birder and Andersson, 2013)**.** The urethral epithelium is supported by the lamina propria, composed of an extracellular matrix containing fibroblasts, adipocytes, interstitial cells, sensory nerve endings and a dense vascular network, which connects the epithelium to the surrounding layers (Birder and Andersson, 2013; Eggermont et al., 2019).

The mucosa at the bladder neck and the proximal urethra contains the majority of LUT nerves (Dixon and Gosling, 1983; Avelino et al., 2002; Birder, 2007). Some epithelial cells lining the urethral lumen show “neuronal-like” properties, including the ability to sense and respond to chemical and mechanical stimuli. This enables them to participate in a complex sensory network alongside nerves and myofibroblasts located

in the underlying layers (Birder and Andersson, 2013; Birder et al., 2014; Birder and Andersson, 2018; Ferreira and Duarte Cruz, 2021). Afferent peptidergic nerves are abundant in the lamina propria, being involved in the detection, processing, and transmission of sensory input originating from the urethral lumen (Barry et al., 2018). Efferent sympathetic and parasympathetic fibres are also present (Grol et al., 2008; Coelho et al., 2010; Eggermont et al., 2019).

Muscle tissues: urethral sphincter

Underlying the mucosa, the urethra is encased in layers of smooth and striated muscle (Figure 4). In the proximal urethra, these muscle fibres are organised to create the urethral sphincter, which is composed of the internal urethral sphincter (IUS) and the external urethral sphincter (EUS) (Pradidarcheep et al., 2011). The contraction of these two units is dependent on distinct mechanisms and includes both excitatory and inhibitory input (Birder et al., 2014; Ferreira and Duarte Cruz, 2021).

The IUS extends between the inferior end of the bladder and the proximal part of the urethra, and it consists of an extension of the detrusor smooth musculature. Smooth muscle cells forming the IUS are slender, thin and arranged in well-defined layers (Pradidarcheep et al., 2011). The contraction of the IUS is controlled by the autonomic nervous system. Sympathetic excitatory input, mediated by noradrenaline (NA) originates at the T10-L2 spinal segments and reaches the IUS via the mesenteric ganglion and hypogastric nerve (Elbadawi and Schenk, 1971). Parasympathetic fibres arise from S2-S4 spinal segments via pelvic nerves. These fibres have a dual role, using NO and ACh as inhibitory or excitatory mediators, respectively. Peptidergic sensory fibres are also present, although they are less abundant in the muscular layer than in the mucosa (de Groat and Theobald, 1976; Purves et al., 2001; Barry et al., 2018; Eggermont et al., 2019).

The EUS forms the outer coating of the muscular wall and is composed of striated muscular cells. In males, the EUS is a distinct structure located below the prostate, surrounding the membranous urethra. In females, the EUS is located just below the bladder neck and comprises both the urethral muscles and the urethrovaginal sphincter (Pradidarcheep et al., 2011). The EUS is mostly controlled by the somatic nervous system, being under voluntary control. The EUS receives excitatory cholinergic input via the pudendal nerve from S2-S4 nerve roots in humans (Zvara et al., 1994), or L6-S1 roots in rodents (Rajaofetra et al., 1992; C. de Groat, 2001; Peng et al., 2006). It is presently unclear if sympathetic and parasympathetic nerves are present. The afferent innervation of the EUS is mediated by sensory peptidergic fibres (Jung et al., 2012; Barry et al., 2018).

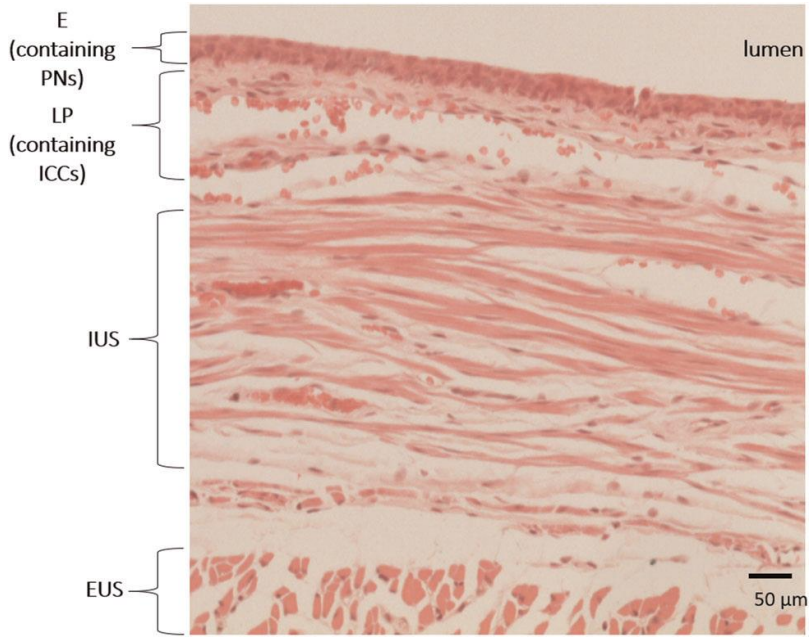


Figure 4. Histological organization of the female urethra. The urethral mucosa is composed of the epithelium (E), which is supported by the lamina propria (LP). The internal urethral sphincter (IUS) is composed of smooth muscle fibres controlled by the autonomic nervous system, followed by the external urethral sphincter (EUS), composed of striated muscle fibres controlled by the somatic nervous system.

Longitudinal section of the proximal portion of the urethra. Scale bars equal 50 µm (adapted from Ferreira and Cruz, 2021).

2.2. The role of the urethra-generated sensory input in efficient voiding

The urine flow along the urethra during voiding activates urethral mechanoreceptive afferent neurons that project to the sacral spinal cord via the pudendal nerve. This input engages spinal and supraspinal reflex circuits, culminating in enhanced bladder contractility. This initiates a positive feedback loop that sustains and strengthens bladder emptying once urination is initiated, enabling efficient voiding (Todd, 1964; Birder et al., 2014). This was first observed in cat and rat models, where this response starts immediately following the onset of urine flow and is maintained well beyond the initial stimulus. Moreover, its amplitude is similar to that of the bladder-to-bladder micturition reflex, and, akin to the latter, requires some degree of bladder filling (Robain et al., 2001; Danziger and Grill, 2015; Danziger and Grill, 2017). It is also known that the electrical stimulation of the urethra triggers detrusor contraction. Depending on the location and stimulation frequency, this stimulation can evoke both inhibitory and excitatory bladder reflexes, which are mediated by distinct pudendal pathways innervating the EUS (Vizzard et al., 1995; Shefchyk and Buss, 1998; Bump, 2000; Gustafson et al., 2003; Peng et al., 2008; Bruns et al., 2009; Woock et al., 2009). These reflexes are suppressed by urethral anaesthesia, further demonstrating the involvement of intraepithelial sensory nerves in LUT function (Jung et al., 1999; Robain et al., 2001; Shafik et al., 2003; Shafik et al., 2007).

Under inflammatory conditions, it is believed that increased excitatory input from the urethra contributes to pain as well as to bladder overactivity, causing symptoms of urgency (Abelli et al., 1991; Nordling et al., 1992; Kamo et al., 2004). In pathologies involving neuronal damage, the neuronal crosstalk between the bladder and urethra

might be affected as well. For instance, in cases of neurogenic detrusor overactivity (NDO) caused by spinal lesion, the electrical stimulation of urethral afferents induced small increases in bladder pressure and decreases in external urethral sphincter activity, yet not sufficient to induce voiding (Shefchyk and Buss, 1998; Bruns et al., 2009; Woock et al., 2009; Kennelly et al., 2010; Yoo et al., 2011; McGee et al., 2017). Thus, in addition to interacting with spinobulbospinal micturition pathways, urethral afferents may have direct access to a spinal circuitry coordinating bladder and sphincter activity.

2.3. The role of Paraneurons in the processing of urethral sensory signals

Paraneurons/paraneuronal cells, also referred to as neuroendocrine cells, are non-neuronal cells recognised as closely related to them, based on their functional properties (Fujita, 1977). Like neuronal cells, paraneurons can produce neurotransmitters, either by synaptic vesicle-like and/or neurosecretion-like granules, in response to stimuli acting upon their receptor site on the cell membrane (Fujita, 1977; Iwanaga and Takahashi-Iwanaga, 2022). Paraneuronal cells are present in several organs, including the stomach, lung, pancreas, urogenital organs, tongue, and skin, among others, where they monitor the external milieu and assist in the regulation and modulation of sensory information within the nervous system (Fujita, 1989).

Urethral afferent nerve fibres, which are located along the lamina propria or muscularis, are not able to reach the luminal surface, where the sensory input originates (Kullmann et al., 2018; Iwanaga and Takahashi-Iwanaga, 2022). Hence, it is believed that urethral paraneuronal cells are the first line of stimulus sensing and processing, integrating afferent information and secreting chemical signals that act on nearby sensory nerves (Birder et al., 2014; Kullmann et al., 2018; Ferreira and Duarte Cruz, 2021; Iwanaga and Takahashi-Iwanaga, 2022). These specialised cells are primarily detected by

their neurotransmitter content, including Ach (Deckmann et al., 2014; Deckmann and Kummer, 2016), somatostatin (Vittoria et al., 1990) or serotonin (5-HT) (Hanyu et al., 1987; Iwanaga et al., 1994; Coelho et al., 2018; Kullmann et al., 2018), with the latter being the most common in urethral tissue. Understanding the role of paraneurons in the urethra might provide important insights into sensory-related pathologies that affect urinary function, such as overactive bladder, bladder pain, and NDO, potentially leading to new therapeutic approaches targeting these neural mechanisms (Iwanaga and Takahashi-Iwanaga, 2022).

2.3.1. Serotonergic paraneurons in the urethral wall

The study of 5-HT paraneurons in the urethra dates from the early '70s, when the first studies identified the presence of 5-HT$^+$ cells dispersed in the urethral epithelium (Owman et al., 1971; Fetissof et al., 1985; di Sant'Agnese, 1986), located near sensory and cholinergic nerve fibres (Tamaki et al., 1992; Iwanaga et al., 2004; Coelho et al., 2018; Kullmann et al., 2018). In some cases, this relationship was pictured through direct contact between the 5-HT$^+$ cells and sensory neurons, further supporting the hypothesis of a neuronal crosstalk between them (Yokoyama et al., 2017; Kullmann et al., 2018).

In the female rat urethra, two morphologically distinct 5-HT$^+$ cell types have been identified. In the mid urethra, 5-HT$^+$ paraneuronal cells are bipolar, featuring elongated, dendrite-like processes that can extend over deeper distances within the epithelial layer. These are likely involved in sensing mechanical stimuli from more distant areas of the urethra, such as the deeper muscular layers. In contrast, in the proximal and distal ends of the urethra, 5-HT$^+$ cells present themselves as multipolar, with shorter processes (Kullmann et al., 2018). These distinct morphological features and anatomical

placements imply that different types of 5-HT^{+} cells detect different stimuli and possibly serve different functions (Kullmann et al., 2018). Interestingly, 5HT^{+} cells are not detected in the bladder, suggesting a restricted role in urethral sensory processing (Coelho et al., 2018).

2.3.2. Functional significance of urethral serotonin

A recent study recording bladder function with urethane-anesthetised isovolumetric cystometry in the female rat demonstrated that the infusion of 5-HT solution into the urethra, but not into the bladder, evoked bladder contractions. Because the bladder-urethral junction was clamped, and therefore fluid exchange between the two organs was not possible, this observation demonstrated that the action of urethral 5-HT in the bladder likely occurs through complex neuronal mechanisms rather than a simple chemical exchange between neighbouring organs (Coelho et al., 2018). During cystometry with urine free flowing through the urethra, 5-HT infusion decreased the frequency of bladder contractions while enhancing their amplitude, indicating that the effect likely happens in response to urethral flow (Coelho et al., 2018). Bladder recordings in tph1 $^{-/-}$ mice, which lack the enzyme responsible for 5-HT production outside the CNS, revealed weaker bladder contractions in the absence of peripheral 5-HT (Coelho et al., 2018).

Following activation by urethral luminal stimuli, serotonergic paraneurons release 5-HT that acts on 5-HT2 and 5-HT3 receptors coupled to urethral afferents. This was demonstrated by co-administration of 5-HT2 and 5-HT3 receptor antagonists, which blocked bladder contractions during 5-HT installation (Coelho et al., 2018). In addition, in vitro electrical stimulation of urethral afferents showed that a large percentage of urethral afferents respond to 5-HT, with increases in Ca^{2+} mediated by 5-HT2 and 5-HT3

receptors (Kullmann et al., 2018). The communication between nerves and 5-HT$^+$ paraneurons is likely bidirectional and operates in positive feedback, as both are immunoreactive for synapsin I, a presynaptic marker. Furthermore, the efferent nerves can release peptides such as calcitonin gene-related peptide (CGRP), substance P, or neurokinin A, that might, in turn, act on paraneurons and further influence 5-HT release (Kullmann et al., 2018) (Figure 5).

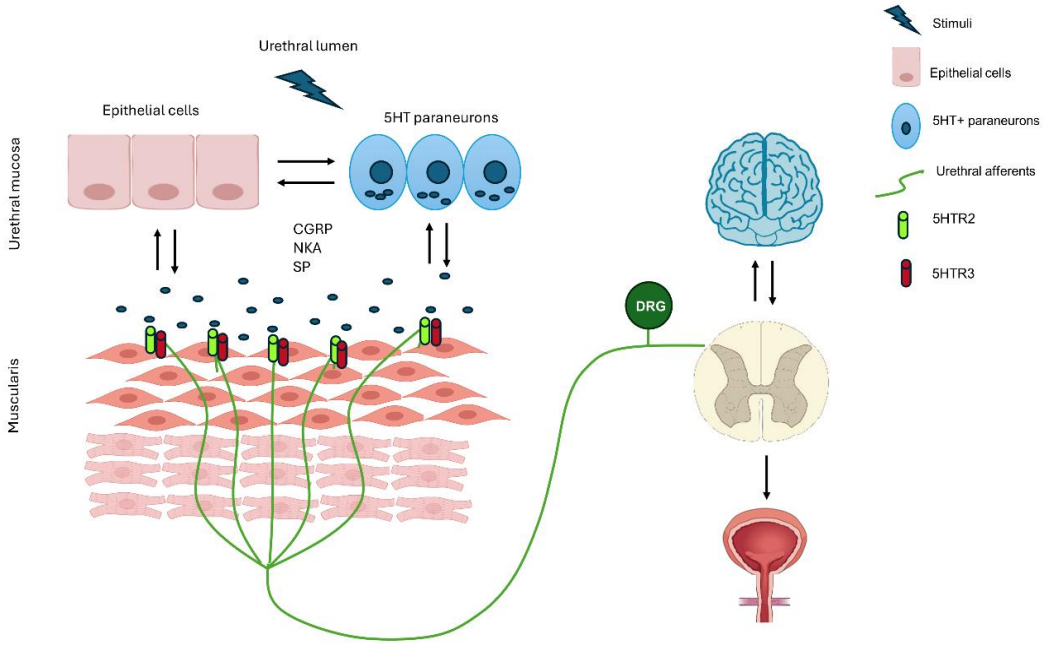


Figure 5. Peripheral 5-HT signalling. The urethral stimuli originate in the urethral lumen and reach the urethral lining, where serotonergic paraneurons are located. In response, these cells release 5-HT that acts on 5-HT2 and 5-HT3 receptors on the afferent nerves present in the lamina propria or muscular layers. The afferent input is then transmitted to the dorsal root ganglia (DRG), spinal cord and supraspinal structures. Descending mechanisms send efferent information back to the LUT, influencing bladder contractions. The communication between nerves and 5-HT$^+$ paraneurons works in positive feedback, as both are immunoreactive for synapsin I. The afferent nerves also release peptides such as Calcitocin-related peptide (CGRP), Substance P (SP) or Neurokin A (NKA) that can activate paraneurones and epithelial cells. Adapted from (Kullmann et al., 2018).

3. LUT dysfunction of neurologic central origin

Considering the high dependency of micturition reflexes on central neuronal mechanisms, it is expected that any damage in the central nervous system, either at the spinal cord or supraspinal structures, would result in some degree of urinary impairment (Panicker et al., 2015; Tish and Geerling, 2020; Ferreira et al., 2023). The region where the lesion occurs is decisive for its clinical manifestations (Panicker et al., 2015) (Figure 3). When injury occurs in regions above the suprapontine region, symptoms reflect the blockade of tonic inhibition of the PMC. This is the case of neuronal damage caused by strokes, neurodegenerative disorders, or traumatic brain injuries, which are typically associated with storage dysfunction and detrusor overactivity symptoms (Blackett et al., 2009; Wyndaele, 2016; Chou et al., 2019; Panicker, 2020; Chiang et al., 2022; Ferreira et al., 2023). If the neuronal injury occurs between the brainstem and the sacrum, as is the case of spinal cord injuries (SCI), spinal stenosis, or neurodevelopment defects like spina bifida, this likely triggers the emergence of an alternative micturition circuit restricted to the spinal cord, independent from supraspinal input (de Groat, 1997; Danzer et al., 2005; de Groat and Yoshimura, 2006, 2009; Ferreira et al., 2023). This alternative reflex induces neurogenic detrusor overactivity (NDO), which the International Continence Society defines as strong involuntary detrusor muscle contractions occurring during filling cystometry in the setting of a clinically relevant neurologic disease (D'Ancona et al., 2019). Opposite to suprapontine lesions, NDO is often concurrent with detrusor sphincter dyssynergia (DSD), which causes urinary incontinence due to a lack of coordination between the bladder and urethra. This is commonly accompanied by dangerously high intravesical pressures that can lead to upper urinary tract deterioration (Ginsberg, 2013). Lesions occurring below the sacrum, such as those caused by

infrasacral SCI, Fowler´s Syndrome, or pure autonomic failure, usually lead to detrusor areflexia or underactivity, resulting in urinary retention and/or urinary incontinence due to loss of urethral sphincter resistance (Panicker et al., 2015; Wyndaele, 2016) (Figure 3). Other pathologies, such as Multiple Sclerosis, can simultaneously generate suprapontine and suprasacral lesions, which worsen urinary dysfunction symptoms (Procaccini et al., 2015).

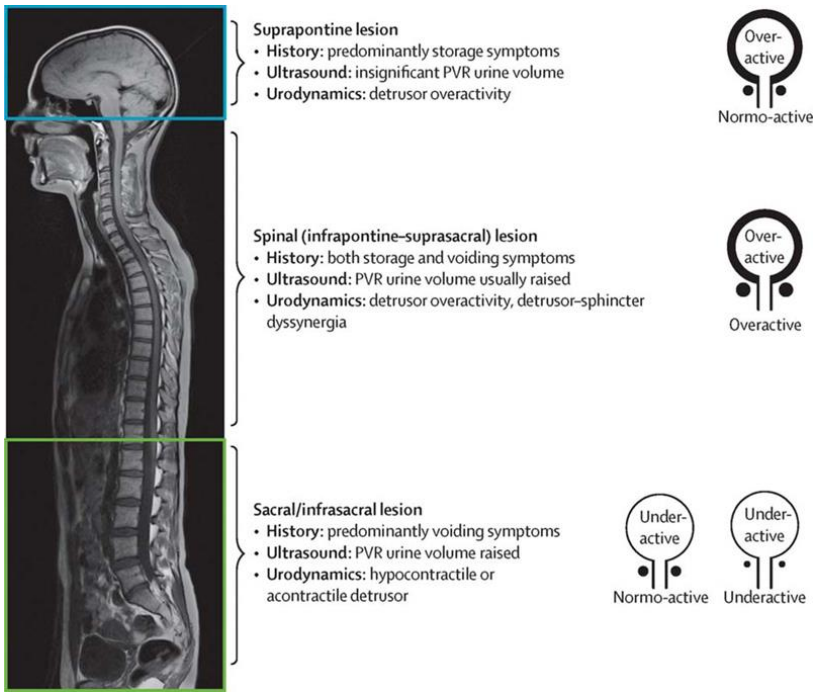


Figure 3. Lower urinary tract dysfunction following neurologic disease according to injury location. Suprapontine lesions induce storage symptoms such as detrusor overactivity and high post-void residual (PVR) volumes. Infrapontine-suprasacral lesions occur between the brainstem and the sacrum and provoke both storage and voiding symptoms caused by neurogenic detrusor overactivity (NDO) and detrusor-sphincter dyssynergia (DSD). Infrasacral injuries cause voiding symptoms, leading to a hypocontractile detrusor and urinary retention and/or urinary incontinence, depending on whether urethral sphincter resistance is lost. Panicker et al., 2015.

4. Spinal cord injury and urinary dysfunction

Communication between peripheral organs and the CNS depends on an intact and fully functioning spinal cord. Hence, damage to the spinal cord can result in significant impairments in multiple bodily systems, depending on the level and severity of the injury. One common example is spinal cord injury (SCI), which disrupts both ascending and descending neural pathways between the brain and regions below the injury location. As a result, SCIs impair motor and sensory functions, as well as the control of some visceral functions, including micturition (Cruz and Cruz, 2011; Hou and Rabchevsky, 2014). Reflecting the level and severity of the injury, most SCI patients experience some degree of urinary impairment, which often leads to the loss of voluntary control over urination (de Groat, 2006). This not only results in urinary complications, including urinary incontinence, recurrent urinary infections and potential kidney damage, but also imposes significant psychological, social, and economic burdens (DeVivo et al., 2011; Simpson et al., 2012; Collinger et al., 2013). Individuals with SCI experience heightened anxiety, reduced self-esteem, and diminished social interactions due to the stigma and practical challenges associated with managing urinary incontinence (Aaby et al., 2020; Budd et al., 2022; Sanguinetti et al., 2022; Calderone et al., 2024).

4.1. Incidence, causes and consequences of spinal cord injuries

In 2019, an estimated 20.6 million people worldwide were living with lifelong consequences of an SCI, with approximately 0.9 million new cases occurring each year (Ding et al., 2022). The majority of cases occur due to traumatic events (Kang et al., 2018), and their incidence varies significantly across age and gender groups, as well as global regions, reflecting diverse socioeconomic and environmental factors. Young males aged 15-29 years have the highest incidence rates, due to vehicle accidents, sports,

violence, or work-related injuries. Conversely, in the elderly, SCI is often provoked by falls associated with neurodegenerative conditions (Chen et al., 2016). Geographically, high-income countries report lower incidence rates due to robust safety regulations and healthcare systems, whereas low-income countries exhibit higher rates, which are exacerbated by occupational hazards, inadequate infrastructure, and limited access to preventive measures (Kang et al., 2018; Ding et al., 2022; Liu et al., 2023). While traumatic SCIs are the most common, non-traumatic aetiologies also contribute to their incidence. Conditions such as congenital spinal cord malformations, spinal tumours, spinal stenosis, myelitis, and other neurodegenerative disorders can all lead to SCI by compromising the structural or functional integrity of the spinal cord (Clark and Marshall, 2017; Müller-Jensen et al., 2021).

The consequences driven by SCI are variable and typically associated with the level and the severity of the spinal injury (Alizadeh et al., 2019; Ding et al., 2022). Cervical and high thoracic segments are the most frequently affected (>50%), followed by thoracic (35%) and lumbar injuries (11%) (Kang et al., 2018; Alizadeh et al., 2019). Most lesions occur due to blunt trauma, where the spinal cord is damaged by a sudden impact (spinal contusion or compression). Less frequently, spinal cord laceration/transection occurs when external objects or fragments of vertebral bone penetrate the spinal cord and directly transect the cord (Kang et al., 2018; Alizadeh et al., 2019).

4.2. Suprasacral SCI-induced alterations in micturition: neurogenic detrusor overactivity and detrusor sphincter dyssynergia

The dependence of micturition on complex neuronal mechanisms involving supraspinal centres renders LUT function particularly vulnerable to SCI. As the

communication between the LUT organs and supraspinal centres is abruptly interrupted, the bladder becomes partially or totally areflexic, with the absence of conscious awareness of bladder filling (Cruz and Cruz, 2011; Wyndaele, 2016). This period is termed spinal shock and can linger for weeks, months or years, causing urinary retention that requires catheterisation (Anderson et al., 2023). As spinal shock progresses, major neuroplastic rearrangements in the lumbosacral spinal cord result in the development of an alternative micturition reflex independent from supraspinal input (de Groat, 1997; de Groat and Yoshimura, 2012). Because micturition is no longer under conscious control, voiding becomes largely inefficient due to neurogenic detrusor overactivity (NDO), often accompanied by detrusor-sphincter dyssynergia (Stoffel, 2016; Furrer et al., 2024). The concurrent presence of NDO and DSD leads to dangerously high intravesical pressures, which is a risk for deterioration of the upper urinary tract and eventual kidney failure (De Groat, 1993; Cruz and Cruz, 2011; Wyndaele, 2016) (Figure 6).

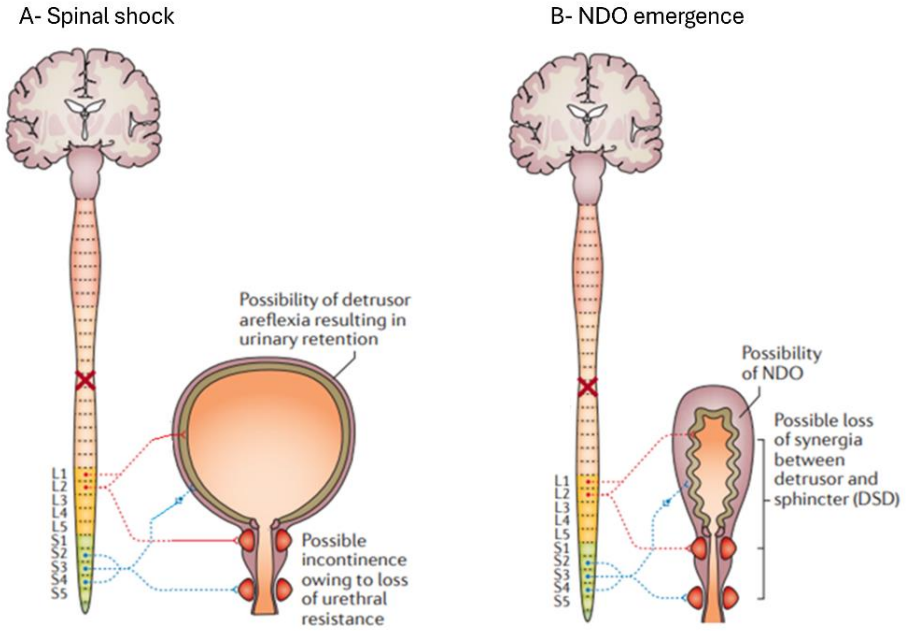


Figure 6. A diagram dissecting the events beyond SCI-induced urinary dysfunction. (**A**) SCI above lumbosacral segments induce the loss of the centralised micturition reflexes and a partially or totally areflexic bladder; (**B**) Neuroplastic events at the lumbosacral spinal cord incite the formation of a new micturition circuit independent from supraspinal centres. The new reflex is characterised by overactivity of the detrusor muscle (NDO) and dyssynergia between detrusor and sphincter contraction (DSD), altogether provoking incontinence symptoms. Adapted from Wyndaele et al., 2016.

4.3. Consequences of spinal cord injuries in LUT organs

The development of NDO in response to SCI triggers noteworthy changes in LUT organs (Cruz and Cruz, 2011). For decades, the bladder was considered to be the main organ involved in NDO pathophysiology. Hence, numerous studies have largely described post-SCI changes in this organ. The bladder undergoes substantial changes in uroepithelial morphology and barrier function. During the acute stage of injury, the urothelium suffers loss of apical umbrella cells, disorganisation of layers, reduction of cellular volume and increased collagen deposition. These changes impair the trans-epithelial barrier function, as tight junctions and cell-cell connections become disrupted (Apodaca et al., 2003; Jiang et al., 2015; Wu et al., 2022; Wyndaele et al., 2024). Following the acute stage, the urothelium undergoes fast recovery, and the barrier function is restored within a few weeks. However, the surface umbrella cells are significantly smaller, indicating a massive turnover of the surface lining cells but with an incomplete degree of differentiation, reflecting a sustained insufficient regenerative ability of the urothelium after SCI (Apodaca et al., 2003; Kullmann et al., 2017; Wyndaele et al., 2024). Because of bladder areflexia induced by spinal shock, bladder capacity significantly increases, implicating massive detrusor muscle hypertrophy and fibrosis (Cruz and Cruz, 2011; Oliveira et al., 2019; Wyndaele et al., 2024).

Bladder sensory fibres, the majority of which are peptidergic C-fibres (Avelino et al., 2002), are the main drivers of NDO emergence (de Groat and Yoshimura, 2010, 2012). Under normal conditions, C-fibres are unresponsive to bladder stretch. After SCI, they undergo massive sprouting and become hyperexcitable due to the increased release within the bladder wall of excitatory mediators, including as prostaglandins, ATP, and NO, by urothelial, smooth muscle and inflammatory cells, as well as interstitial cells of Cajjal (Masunaga et al., 2006; Johnston et al., 2008; Smith et al., 2008). Increased exposure to these mediators leads to bladder afferent hyperexcitability, and activation of bladder afferents by low-level bladder distension leads to detrusor contraction and NDO. Autonomic fibres are also damaged, particularly in the detrusor muscle, where parasympathetic denervation has been detected (Drake et al., 2010; Johnston et al., 2012). Given the importance of the urethra in micturition reflexes (Ferreira and Duarte Cruz, 2021), it is likely that SCI-induced voiding dysfunction also induces alterations in urethral histology and innervation, but this remains a poorly investigated area.

4.4. Management of LUT dysfunction after spinal cord injury

Presently, no treatment fully resolves urinary dysfunction associated with SCI. The standard of care comprises a combination of therapies designed to alleviate symptoms, with a primary focus on improving the patient's quality of life. During spinal shock, as the bladder is areflexic and urine retention is almost complete, the primary concern is to drain urine to prevent the rise of intravesical pressure and protect the upper urinary tract (Wyndaele, 2016). Therefore, catheterisation is needed and typically achieved by an indwelling catheter until the diuresis and cardiovascular functions are stable. When appropriate, this approach can be replaced by clean intermittent catheterisation later on (Wu et al., 2022; Sartori et al., 2024).

In the absence of preventive measures that impede or reverse the development of NDO and DSD, urinary incontinence may become a detrimental condition that accompanies SCI. The standard of care for incontinence symptoms consists of the combination of intermittent catheterisation and oral antimuscarinic drugs, which block M2 and M3 receptors present in the detrusor and prevent acetylcholine-mediated detrusor contractility (Panicker et al., 2015; Wyndaele, 2016). The most used antimuscarinic drugs are oxybutynin, trospium chloride, tolterodine, propiverine, darifenacin, and solifenacin (Sartori et al., 2024). Even though these drugs can effectively reduce incontinence episodes, they are accompanied by significant side effects, such as constipation, dry mouth, blurred vision, tachycardia, and even cognitive impairment, prompting patients to discontinue treatments (Wyndaele, 2016; Sartori et al., 2024). β3-Adrenergic receptor agonists such as mirabegron and vibegron were shown to improve symptoms in NDO patients, but deeper investigation is needed to establish their exact role and optimal dosages (Sartori et al., 2024). In refractory patients resistant to pharmacologic treatment, injections of BoNT/A (Type A Botulinum neurotoxin) in the detrusor muscle are the current gold standard option (Panicker et al., 2015). This neurotoxin acts by blocking the vesicle-mediated neurotransmission in the detrusor muscle, causing chemical denervation of cholinergic, adrenergic, and peptidergic fibres (Coelho et al., 2010; Coelho et al., 2012). This treatment effectively reduces incontinence episodes but must be repeated every 8-10 months (Cruz et al., 2011; Weckx et al., 2016). Some patients present a gradual loss of the therapeutic benefit after multiple injections, while others may continue to respond effectively to the toxin over a prolonged period (Hebert et al., 2020). BoNT/A detrusor injections are not exempt from side effects, which include high post-void residual urine and frequent urinary tract infections (Weckx et al.,

2016; Panicker, 2020; Sartori et al., 2024). More invasive therapeutic protocols include surgical bladder augmentation, urinary diversion, bladder neck reconstruction, sphincterotomy or implantation of artificial urethral sphincters, or sacral neuromodulation (Wyndaele et al., 2018; Sartori et al., 2024).

5. Animal models of SCI

While advancements in non-animal models, such as organoid systems or computational simulations, have revolutionised biomedical research, these still cannot replicate the complexity of the systemic interactions in a living organism, remaining more useful to complement than to replace animal models (Mukherjee et al., 2022). In SCI research, animal models are still indispensable to understand the complexity of the condition and its widespread effects on multiple body systems (Sharif-Alhoseini et al., 2017). Over the years, animal models of SCI have provided valuable insights into the pathophysiology of the disease and allowed the testing of multiple therapies that greatly improved the mortality and morbidity among human patients (Sharif-Alhoseini et al., 2017; Ferreira et al., 2023).

5.1. Animal Species

Rodents are the most commonly used organisms to induce experimental SCI (Sharif-Alhoseini et al., 2017; Ferreira et al., 2023). Rats have the advantage of ease of care and a well-characterised physiology (Fry et al., 2010). Its bigger body and spinal cord size, when compared to smaller rodents, allow more complex and precise injury modelling, which is critical for models based on the physical lesioning of specific areas of the spinal cord. In addition, most urodynamic recording procedures are better studied and implemented in rats, providing more robust and sensitive tools for urodynamic

evaluation (Sartori et al., 2021). Its longer lifespan (2-3 years) allows longer-term studies, which are sometimes challenging when using mice. Nonetheless, mouse models are becoming more popular in SCI research, as they allow the generation of genetically modified animals and the study of specific genes in disease mechanisms and repair (Sharif-Alhoseini et al., 2017; Ferreira et al., 2023). In addition, mice have higher reproductive rates and lower maintenance costs than rats, which further benefits their use. Nevertheless, mice's small size is a problem for surgical protocols, as well as for urodynamic recordings and urethral electromyography (Andersson et al., 2011). Larger animal models, such as rabbits, pigs or non-human primates, are highly valuable due to their larger size and neuroanatomical and physiological similarities to humans, which provide an unmatched translational potential. However, their longer maturation time, complex housing with associated costs, but, above all, the ethical restrictions remain challenges constraining their use (Sharif-Alhoseini et al., 2017; Ferreira et al., 2023).

5.2. Sex

Contrary to what happens in the clinical setting, where the majority of SCIs affect men, experimental SCIs are frequently induced in female experimental animals. This is related to their simple LUT anatomy, with a shorter urethra and absence of the prostate, which simplifies transurethral catheterisation and abdominal compression for urine removal during spinal shock (Pradidarcheep et al., 2011). However, gender dysmorphism in micturition behaviour should be accounted for in data interpretation. Male rodents exhibit more variable micturition patterns due to the influence of testosterone (Saad et al., 2011). While hormonal cycles in females can also induce changes in bladder activity (Longhurst and Levendusky, 2000; Pan and Malykhina, 2014), their effects are easier to account for and female animals tend to have more predictable voiding behaviour (Lovick

and Zangrossi, 2021). However, gender dysmorphism in micturition goes way beyond hormonal effects, as there is evidence of neurophysiological differences between the two sexes. The cholinergic neurotransmission is predominant in the male bladder, while the purinergic component is prevalent in females (Patra and Patra, 2012). Sex differences are also documented in the expression of acid-sensitive ion channels (ASICs) and transient receptor potential vanilloid type 1 (TRPV1), where females have a significantly higher expression of both key channels (Kobayashi et al., 2009). Functionally, male voiding consists of a fast spike-like urine flow, whereas in the female, voiding is ongoing but interrupted for short periods when bladder pressure is increased. In addition, the maximum flow rate is lower and the voiding period is shorter in females as compared to male rats (Streng et al., 2002). These dimorphic patterns could reflect differences in the perineal muscles of the EUS, which are less prominent in females (Cruz and Downie, 2005). Though these differences are considered to be of minor relevance in normal function, they might have a significant impact on pathological conditions.

5.3. Type of injury

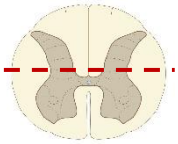
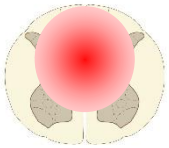
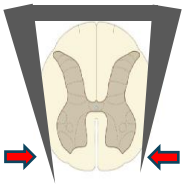
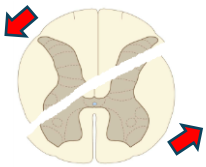
The majority of the animal models used to study SCI are based on the traumatic lesion of the spinal cord by complete spinal cord transection, followed by spinal hemisection. Less used are the contusion, compression, or spinal cord displacement models (Table 1) (Ferreira et al., 2023). These models are usually inflicted on low thoracic regions (Kramer et al., 2017; Ferreira et al., 2023), as cervical and high thoracic injuries often result in respiratory compromise and increased mortality in animal models (Lane et al., 2008; Jeffries and Tom, 2021; Rodgers et al., 2022). This contrasts with human pathology, where cervical or high thoracic segments are the most frequently affected segments (Sekhon and Fehlings, 2001).

Spinal cord transection (SCT) is the most commonly used method to study SCI-induced NDO (Ferreira et al., 2023). This experimental method is simple, economical, and highly reproducible (Blight, 2000; Lee et al., 2013; Cheriyan et al., 2014). The transection can be complete or incomplete, allowing targeting the lesioning of specific regions. In Humans, spinal cord sectioning occurs due to spinal cord laceration provoked by displaced fragments of bone or tissue. Nevertheless, transection injuries are rarely seen in clinical practice and thus do not truly reflect the pathophysiology of SCI (Cheriyan et al., 2014; Theodore, 2016; Liu et al., 2019).

Spinal cord contusion (SCC) results from an impact in the spinal tissue with a variable force, which is meant to mimic human SCI caused by blunt trauma caused by abrupt movements of the head, neck or back (Sharif-Alhoseini et al., 2017; Verma et al., 2019). In those cases, lesions are often incomplete and may affect specific regions of the cord, causing inflammation, ischemia, hemorrhagic necrosis and central cavitation (Blight, 2000; Sharif-Alhoseini et al., 2017; Verma et al., 2019). Experimental contusion protocols are often based on the weight drop method (Allen, 1911), which consists of dropping a mass from a specific height onto the dorsal surface of the exposed spinal cord. Over the years, adaptations of the pilot protocol resulted in the generation of commercially available automated contusion devices (New York University (NYU) impactor and Infinite horizon impactor, for instance), which decreased variability by producing a force- and dwell-timed controlled impact (Sharif-Alhoseini et al., 2017; Verma et al., 2019). Despite closely resembling human SCIs, contusions carry considerable inter-trial variability, which often translates into an increase in the number of animals necessary to reach significant conclusions (Cheriyan et al., 2014; Sharif-Alhoseini et al., 2017).

Spinal cord compression, using aneurysm clips or ischemia balloons, simulates the spinal canal occlusion resulting in the SCI-induced ischemic environment seen in some human injuries. Moreover, they are preferred for studying the optimal timing and surgical processes of spinal decompression. Dislocation or distraction spinal cord models cause SCI by lateral displacement of vertebrae, applying opposing traction forces to stretch the spinal cord. Less frequently, SCI can also be induced using ischemic, photochemical, and electrolytic animal models (Sharif-Alhoseini et al., 2017).

Table 1. Animal models of traumatic Spinal Cord Injury. Description of injury models to mimic human SCIs, focusing on their advantages and disadvantages. Based on Ferreira et al. 2023.

Injury model	Method induction	Advantages	Disadvantages
Transection 	Total or partial cord sectioning with scissors or scalpel causing massive bleeding.	-Easy to induce; -Cheap; -Highly reproducible; -Allow lesioning of specific tracts if using hemisection.	-Poor translational value; -Does not allow injuries of different severities.
Contusion 	Blunt trauma caused by a controlled weight drop, causing ischemia, inflammation, hemorrhagic necrosis and central cavitations.	- Clinically relevant -Allow lesioning of specific tracts (if using hemicontusion) - Allow induction of injury of different severities by varying the height of the drop or weight mass	-Technically difficult; -Highly costly; -Low reproducibility.
Compression 	Compression of the spinal cord using balloons, forceps or aneurysm clips provoking ischemia.	- Closely resembles human SCIs; -Allow induction of injury of different severities by varying time and/or force of compression.	-Technically difficult; -Does not allow lesioning of specific tracts; -Highly costly; -Low reproducibility.
Dislocation 	Two beams are attached to adjacent vertebrae, causing lateral displacement of the vertebra and inducing ischemia.	-Clinically relevant -Allow indication of injury of different severities by varying time and/or displacement velocity.	-Technically difficult; -Does not allow lesioning of specific tracts; -Low reproducibility.

5.4. SCI models used in the study of NDO

Animal models used in the investigation of SCI-induced urinary impairments have largely relied on complete or incomplete spinal cord transection paradigms (Ferreira et al., 2023) (Figure 8). These remain easy-to-induce and highly reproducible models, with a well-established and predictable phenotype of urinary dysfunction, having allowed the clarification of pathophysiological mechanisms and the development of new therapeutic strategies. Still, transection injuries are rare among human SCIs. Hence, there has been an effort to implement contusion models in urological research, as they closely represent human pathology and probably induce a more accurate phenotype of urinary dysfunction (Ferreira et al., 2023).

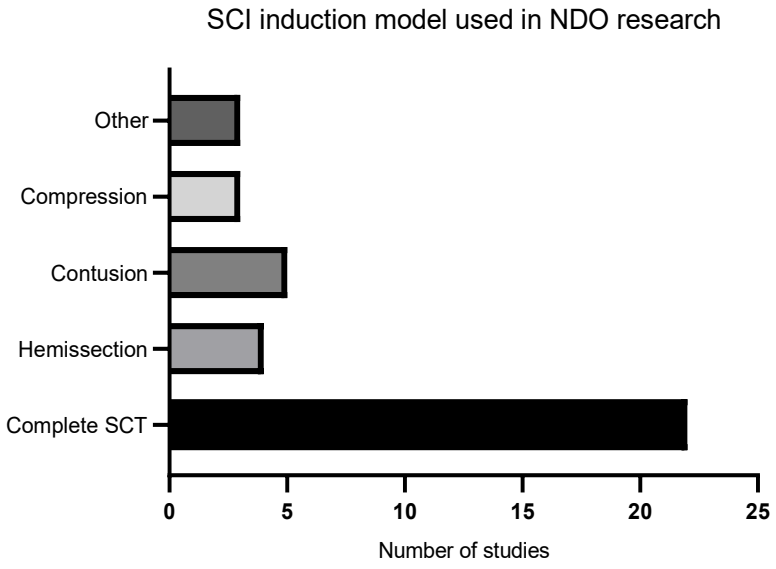


Figure 8. **Animal models used to inflict Spinal Cord Injury-urinary dysfunction**. Most SCIs are induced by complete spinal cord transection (SCT). Less used models include spinal hemisection, contusion, compression, and other models. Data was obtained through a systematic review focusing on animal models of SCI used in the study of Neurogenic Detrusor Overactivity (NDO). Adapted from Ferreira et al., 2023.

As most past studies have relied on transection models, at present, it is not clear if urinary impairments arising after transection and contusion injuries are comparable, raising the question of whether the results observed in a specific model can be extrapolated to another, and later to the clinical context. Typically, lesions associated with complete transection of the spinal cord are associated with a higher level of urinary retention during the spinal shock, besides taking longer to develop reflexive voiding, when compared to contusion injuries (Leung et al., 2007; Breyer et al., 2017). However, some results seem contrasting, as urodynamic data collected from animals left to recover for four or more weeks after SCI showed that the frequency and amplitude of voiding contractions do not significantly vary across injury models (Mitsui et al., 2014; Breyer et al., 2017). This could suggest that, despite different degrees of damage to the spinal cord, injuries result in similar functional impairment of the lower urinary tract (Pikov et al., 1998; Pikov and Wrathall, 2001; Breyer et al., 2017). Nonetheless, it is also possible that the molecular mechanisms leading to such impairments may differ. Studies concerning changes in LUT innervation and morphology after different SCI models are scarce (Mitsui et al., 2014) and only address the urinary bladder and rarely the urethra, an important LUT organ. The effects of different SCI models in lumbosacral spinal cord innervation, to where LUT afferents project and are considered a major hub for post-SCI neuroplasticity, were also never assessed. These points should be better understood to define the mechanisms leading to NDO emergence in different SCI models and eventually fine-tune the testing of new therapies.

Goals

Lower urinary tract (LUT) functions are highly dependent on supraspinal input, which is jeopardised after insults occurring in the central nervous system. These insults can result from a variety of pathologies, including spinal cord injuries (SCI). Typically, SCIs are followed by a period of little to no bladder activity. After this period, termed “spinal shock”, SCI is followed by a complex cascade of maladaptive neuroplastic events that lead to the emergence of an alternative micturition reflex, independent from brain input, ultimately leading to urinary incontinence and other complications. Research on post-SCI urinary impairment has predominantly relied on the use of animal models of complete spinal cord transection, given their simplicity and high reproducibility rates. However, such injuries are uncommon in humans, which creates the need for a shift towards more clinically relevant contusion models. Whether different animal models of SCI result in similar patterns of urinary dysfunction and neuroplastic rearrangements in LUT and lumbosacral spinal cord remains unclear. Moreover, while evidence suggests that bladder function reflects an active and efficient crosstalk between the bladder and the urethra in spinal-intact animals, little is known about the consequences of SCI to the urethra and the urethro-vesical neuronal crosstalk.

As such, the present thesis had the following goals:

Goal 1. To systematically review the use of animal models for the study of LUT dysfunction of central neurologic injury (***Publication I***).

Goal 2. To describe variations in SCI-induced urinary impairments associated with different models of SCI in the female rat (spinal cord transection versus spinal cord contusion) (***Publication II***):

a) *To describe the effects of spinal cord transection and different severities of spinal cord contusion on bladder function.*

b) *To quantify the consequences of the different SCI models on LUT and lumbosacral spinal cord (L5-S1 spinal segments) innervation*.

Goal 3. To document SCI-induced alterations in urethral tissues, identifying the consequences of spinal cord transection to urethral histology and innervation in the female rat at different time points of disease progression (***Publication III***).

Goal 4. To study the consequences of SCI-induced urethral changes to the urethro-vesical neuronal crosstalk mediated by peripheral serotonin (5-HT) in female mice (***Publication IV-*** *manuscript submitted to Scientific Reports*):

a) *To describe the effects of spinal cord transection on bladder function of wildtype (WT) and transgenic mice lacking peripheral expression of tryptophan hydroxylase 1, the enzyme necessary for peripheral serotonin production (tph1$^{-/-}$ mice).*

b) *To document the consequence of SCI on the number of 5-HT^{+} paraneuronal cells lining the urethra, as well as its effects on SCI-induced urethral tissue rearrangement in WT and tph1$^{-/-}$ mice.*

c) *To explore the therapeutic potential of serotonin modulation in the SCI-bladder function, by early and sustained treatment with 5-HT receptor antagonists.*

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Publications

Publication I

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Review

Molecular Mechanism Operating in Animal Models of Neurogenic Detrusor Overactivity: A Systematic Review Focusing on Bladder Dysfunction of Neurogenic Origin

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Abstract: Neurogenic detrusor overactivity (NDO) is a severe lower urinary tract disorder, characterized by urinary urgency, retention, and incontinence, as a result of a neurologic lesion that results in damage in neuronal pathways controlling micturition. The purpose of this review is to provide a comprehensive framework of the currently used animal models for the investigation of this disorder, focusing on the molecular mechanisms of NDO. An electronic search was performed with PubMed and Scopus for literature describing animal models of NDO used in the last 10 years. The search retrieved 648 articles, of which reviews and non-original articles were excluded. After careful selection, 51 studies were included for analysis. Spinal cord injury (SCI) was the most frequently used model to study NDO, followed by animal models of neurodegenerative disorders, meningomyelocele, and stroke. Rats were the most commonly used animal, particularly females. Most studies evaluated bladder function through urodynamic methods, with awake cystometry being particularly preferred. Several molecular mechanisms have been identified, including changes in inflammatory processes, regulation of cell survival, and neuronal receptors. In the NDO bladder, inflammatory markers, apoptosis-related factors, and ischemia- and fibrosis-related molecules were found to be upregulated. Purinergic, cholinergic, and adrenergic receptors were downregulated, as most neuronal markers. In neuronal tissue, neurotrophic factors, apoptosis-related factors, and ischemia-associated molecules are increased, as well as markers of microglial and astrocytes at lesion sites. Animal models of NDO have been crucial for understanding the pathophysiology of lower urinary tract (LUT) dysfunction. Despite the heterogeneity of animal models for NDO onset, most studies rely on traumatic SCI models rather than other NDO-driven pathologies, which may result in some issues when translating pre-clinical observations to clinical settings other than SCI.

Keywords: animal models; lower urinary tract dysfunction; neurogenic bladder; neurogenic detrusor overactivity; detrusor sphincter dyssynergia

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1. Introduction

The lower urinary tract (LUT), comprising the urinary bladder and urethra, is responsible for the storage and periodic elimination of urine. Micturition relies on the synchronized activity of the bladder and the urethral sphincter, the functional muscular unit that controls urine flux [1,2]. Thus, the expulsive contractions of the detrusor muscle are tightly coordinated with relaxation of the urethral sphincter, to ensure efficient urine removal. Normal LUT function relies on complex networks involving neurons operating in an on–off switch-like manner and located in the peripheral ganglia, spinal cord, and supraspinal centers [1,3]. These neuronal circuits are established and matured during infancy when voluntary control over micturition is learned. Activation of these circuits allows conscious and voluntary switching from storage to voiding, influenced by the perceived state of bladder fullness and assessment of the social appropriateness [1]. Therefore, the complexity of neuronal

LUT control is such, that it comes as no surprise that voluntary control over micturition is easily jeopardized in neurologic conditions affecting the central nervous system (CNS), including spinal cord injury (SCI), stroke, and progressive neurodegenerative disorders, such as Parkinson's disease (PD) or multiple sclerosis (MS) [4–6].

The most common urinary dysfunction arising from central neurologic disease is neurogenic detrusor overactivity (NDO), defined by the International Continence Society as "Involuntary detrusor muscle contractions that occur near or at the maximum cystometric capacity, in the setting of a clinically relevant neurologic disease. These contractions generally cannot be suppressed, resulting in urinary incontinence or even reflex bladder emptying (reflex voiding)." [7]. The region where the lesions occur is pivotal for its clinical manifestations. When NDO arises from damages in suprapontine areas (e. g. stroke, PD), symptoms reflect the blockade of tonic inhibition of the pontine micturition center, resulting from damages to supraspinal micturition pathways. These damages are mostly associated with storage symptoms, particularly manifested as detrusor overactivity, as a result of bladder outlet obstruction and urethral sphincter dysfunction [8,9].

If the injury occurs due to damages in the suprasacral spinal cord (e.g., SCI), this triggers the emergence of alternative micturition pathways, totally located at the lumbosacral spinal cord, operating in the absence of supraspinal input, and dependent on afferent C fibers [10–12]. Unlike what happens after suprapontine lesions, in this case NDO is often concurrent with detrusor sphincter dyssynergia (DSD), resulting in impaired bladder emptying, high residual volumes of urine and frequent episodes of urinary incontinence [5]. There are also cases when the damages may originate from both suprapontine and suprasacral lesions in diseases such as MS, for example. NDO is also likely to occur due to neurogenic inflammation of the bladder, when a physical interruption of brain–bladder circuits is not evident [13,14].

Pharmacological NDO treatment aims to reduce detrusor contractions and promote continence. It is initiated with anticholinergic drugs, with intradetrusor injections of botulinum toxin A remaining the gold standard option for refractory patients [15]. Pharmacological interventions are combined with intermittent catheterization, performed by the patient or the caregiver [16]. NDO courses have increased frequency of urinary tract infections and the risk of kidney deterioration is high [17]. Therefore, health and quality of life of NDO patients is severely compromised. Moreover, available therapies often carry significant side effects, and some may lose efficiency over time. Therefore, a breakthrough in the treatment of NDO is urgently needed. To produce a significant advance in NDO therapies, it is necessary to gain a better understanding of NDO pathophysiology and grasp molecular changes that underly this pathology, including changes in the expression of many receptors, trophic factors, and inflammatory mediators. Animal models have been critical for this, as they offer the possibility to investigate in vivo consequences of NDO, including shifts in urodynamic parameters, and identify changes in gene expression and electrophysiological properties of neurons involved in the control of LUT function [6,11]. Furthermore, by mimicking human pathology, the back translational value of animal models becomes more evident, as they allow the direct testing of new drugs and therapies before advancing research to clinical trials.

There are many studies using animal models of NDO but published results are diverse and difficult to interpret in an integrative manner. However, to propose truly innovative hypotheses, it is necessary to congregate data and critically review published results, appreciating the wealth of knowledge generated. Therefore, the present systematic review aims to collect and discuss pre-clinical literature focusing on molecular changes associated with NDO of central origin, providing an up-to-date analysis of molecular studies involving animal models to assess NDO published in the last decade. This review seeks to present a holistic view of the current findings in animal NDO models, which is essential to shape future research directions in the field and propel clinical translation findings.

2. Results

The fine molecular mechanisms involved in NDO emergence and maintenance are currently better understood [18], but remain challenging for clinicians and researchers. A fully effective treatment, able to revert urinary dysfunction, remains to be identified and there is ample need to improve symptomatic care for NDO patients and, as a consequence, their quality of life. Current treatment aims primarily to protect the upper urinary tract and, later on, to promote continence. However, treatments are not fully effective, carry bothersome secondary effects, such as cognitive impairment (due to prolonged use of anti-muscarinic drugs) and increased frequency of urinary infections, and are not able to revert loss of control over bladder function [19,20]. Therefore, animal models of NDO are critical to clarify NDO pathophysiology and pinpoint putative therapeutic targets.

The database search used the key words ((Animal model) OR (rat) OR (mice) OR (rabbit) OR (pig)) AND ((Neurogenic detrusor overactivity) OR (neurogenic bladder)) and identified 648 candidate studies. After the removal of duplicate records, 365 studies were screened by title and abstract. Two hundred and thirteen records were excluded: 129 were unrelated studies, 13 studies were published in languages other than English, and 20 records focused on human patients as their study population. Fifty-one publications were also excluded because they were not journal articles, and included reviews (n = 42), conference papers (n = 2), and comments or editorials (n = 7). From the remaining 152 studies, it was not possible to retrieve 5 publications. The full texts of 147 articles were screened according to the inclusion and exclusion criteria (see below), which resulted in 51 eligible studies for this systematic review. From the 147 screened articles, 2 reports were excluded, as they used aging as an NDO model but failed to indicate neuronal causes for this urinary dysfunction. Finally, 6 publications were further dismissed, as they used spinal cord injury (SCI) as an NDO model but only focused on early-stage SCI, i.e., 14 days after spinal injury, when animals are still in spinal shock [21–23] and there are little or no signs of bladder reflex activity [24–27]. They also failed to produce urodynamic data indicative of NDO within that time frame. The study selection process is depicted in Figure 1, and a summary of the included studies is presented in Table 1.

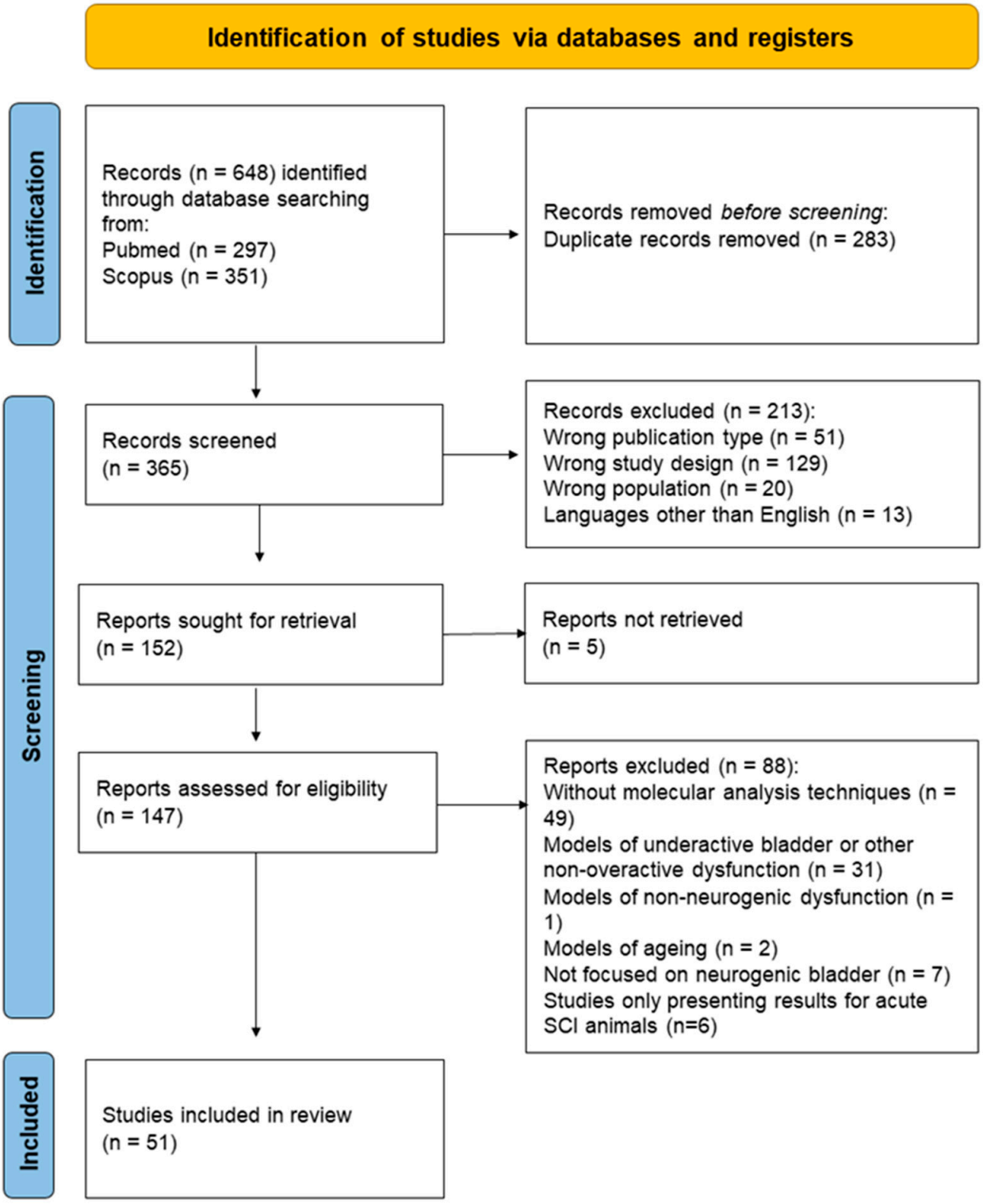

Figure 1. PRISMA flow diagram with the screening process for the present systematic review.

Table 1. Summary of included studies in this systematic review. The data were extracted and sorted into the following categories: model, species, sex, induction method, urodynamic findings, changes in bladder tissue, changes in neuronal tissue, and therapies/mechanisms identified. NDO: neurogenic detrusor overactivity; SCI: spinal cord injury; MAG: myelin-associated glycoprotein; OMpg: outer membrane protein G; RGMa: repulsive guidance molecule A; NGF: nerve growth factor; DRG: dorsal root ganglion; Trka: tropomyosin receptor kinase A; AKT: protein kinase B; TRPM4: transient receptor potential cation channel subfamily member 4; NF200: neurofilament 200; S100: S-100 protein marker; TRPV1: transient receptor potential cation channel subfamily V member 1; M3: muscarinic receptor 3; TGFB1: transforming growth factor beta 1; BoNT-A: botulinum toxin-A; DSD: detrusor sphincter dyssynergia; BDNF: brain-derived neurotrophic factor; ASIC: acid-sensing ion channel; RTX: resiniferatoxin; GAP43: growth-associated protein 43; CGRP: calcitonin gene-related peptide; IPHFO: duration of intra-luminal pressure at high-frequency oscillations; GFAP: glial fibrillary acidic protein; PGE2: prostaglandin E2; HIF: hypoxia-inducible factor; TGF: transforming growth factor; bFGF: basic fibroblast growth factor; NVC: non-voiding contraction; CRF: corticotropin-releasing factor; EUS: external urethral sphincter; PGP. 9.5: protein gene product 9.5; TH: tyrosine hydroxylase; JNK: c-Jun N-terminal kinase; SNAP-25: synaptosomal-associated protein, 25kDa; ATF3: activating transcription factor 3; TRPA1: transient receptor potential cation channel subfamily A member 1; P2X: purinergic receptors; PTNS: percutaneous tibial nerve stimulation; 5-HT: 5-hydroxytryptamine; VGLUT2: vesicular glutamate transporter 2; GABA: gamma-aminobutyric acid; GAD2: glutamate decarboxylase 2; KV1.4: potassium channel Kv1.4; CD11: integrin beta chain-2; SMA: smooth muscle actin; ki67: antigen KI-67; TUNNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling; ICC: interstitial cells of Cajal; PAG: periaqueductal gray; PMC: pontine micturition center; GAPDH: Glyceraldehyde-3-Phosphate dehydrogenase; TGN: tissue gene nerve; VEGF: vascular endothelial growth factor; iNOS: nitric oxide synthase; VTA: ventral tegmental area; IFN: interferon; MBP: myelin basic protein; CTGF: connective tissue growth factor; TGF: transforming growth factor; EAE: experimental autoimmune encephalomyelitis; CNS: central nervous system; IL-B: interleukin beta; CIE: coronavirus-induced encephalitis; MS: multiple sclerosis; Panx1: pannexin 1; cx34: gap junction alpha-1 Protein; SCF: Skp1-cullin 1-F-box; EP2/3: prostaglandin receptors; MCAO: experimental middle cerebral artery occlusion; PD: Parkinson´s disease; 6-OHDA: 6-hydroxydopamine; MPTP: 1-metil-4-fenil-1,2,3,6-tetraidropiridine; VaCHT: vesicular acetylcholine transporter; MMC: meningomyelocele; NeuN: neuronal nuclei.

Study	Model	Species	Sex	Induction Method	Urodynamic Findings	Changes in Bladder Tissue	Changes in Neuronal Tissue	Therapies/Mechanisms Identified
Chambel et al., 2022 [28]	SCI	Rat	Female	Complete cord transection T8/T9	Cystometry under urethane anesthesia: at 7 dpi, bladder contractions were abolished, while at 28 dpi, the NDO was established, with the parameters of frequency, amplitude, basal pressure, and peak pressure augmenting in comparison with intact rats.		Changes in lumbosacral expression of axonal growth regulators: neurocan, phosfacan, nogoA, MAG, OMgp, RGMa: Increase at 7 dpi and decrease to basal levels at 28 dpi -Decrease in the DRG of those specific receptors: CSPG- and MAI-receptors.	NGF regulates the expression of receptors for axonal guidance cues in DRG neurons. When exposed to high levels of NGF, some repulsive cues decrease its expression.
Zhang et al., 2019 [29]	SCI	Rat	Female	Complete cord transection T8/T9 at suprasacral levels		After acupuncture treatment: -Decrease in bladder weight; -Decrease in fibrosis levels and smooth muscle cell damage; -Increase in NGF and TRka and p-akt with treatment: nerve regeneration.		Sacral electroacupuncture therapy can improve the expression of both NGF/TrkA signaling and AKT signaling in the local nerve of the damaged spinal cord and is capable of improving micturition function after injury.

Table 1. *Cont.*

Study	Model	Species	Sex	Induction Method	Urodynamic Findings	Changes in Bladder Tissue	Changes in Neuronal Tissue	Therapies/Mechanisms Identified
Kullmann et al., 2018 [30]	SCI	Mouse	Female	Complete cord transection at T8–T9		-TRPM4 levels largely increase after SCT—earlier time points 3–7 days; -Blocking of TRPM4 activity (by an antagonist) reduces spontaneous muscle activity.		Pharmacological blocking of TRPV4 (9-phenanthrol-ANTAGONIST) reduces spontaneous phasic activity developed after SCI, showing its involvement in SCI-induced bladder neurogenic activity.
Gotoh et al., 2020 [31]	SCI	Mouse	Female	Complete cord transection T8–T9	Awake cystometry: decreased number of non-voiding contractions in vibregon treated rats; the time required to induce the first voiding was higher.	Increase in mRNA levels of collagen, TGF-B1, FGF-2, HIF-a, VEGF-a (ischemic molecules); Vibregon treatment decreased the levels of collagen, TGF and HIF; collagen protein levels were not altered.		Vibregron (β3-adrenoceptor agonist) treatment reduces non-voiding contractions and decreases the expression levels of fibrosis- and ischemia-related molecules.
Chung et al., 2015 [32]	SCI	Rat	Male	Complete transection, or cord compression	Awake cystometry: injured animals presented detrusor overactivity, showing frequent non-voiding contractions; treatment with inosine reduced the frequency and amplitude of detrusor contractions.	-Decreased expression of synaptophysin (SYP); -Decreased expression of NF200-positive axons in bladder; -Increased TRPV1 expression. Inosine treatment attenuated these alterations.		Inosine treatment (immediate or delayed) reduces urinary complications after SCI. The mechanisms are unclear, but it is known that it is not related to muscle contractility, and maybe is through TRPV1.
Shang et al., 2019 [33]	SCI	Rat	Female	Complete transection at T9–T10	Awake cystometry: increased basal pressure,maximum voiding pressure, threshold pressure, volume of residual urine, and number of voiding contractions; decreased inter-contraction interval. All features attenuated by treatment.	Decreases expression of M3 receptor mRNA in SCI animals, and treatment decreased this expression; M3 receptor protein expression does not correlate with changes in the level of M3 receptor mRNA.		Downregulation of M3 receptor expression in the bladder wall by lentivirus-mediated RNAi can significantly decrease urinary dysfunction, targeting NDO symptoms associated with SCI.
Jia et al., 2021 [34]	SCI	Rat	Female	Complete transection at T10	Awake cystometry: increase in non-voiding contractions; botox attenuated this, more in earlier treatments than late.	-Increase in bladder weight, levels of connective tissue/fibrosis; -TGF-β1 (TGF-β1 is a major driver of human fibrotic protein) expression increased.Treatment with BoNTA decreased significantly in the early group compared with the late group; mRNA levels were in line with this.		Early BoNTA injection had a tendency to prevent bladder fibrosis and improve NDO, downregulating TGF-β1 expression.
Saito et al., 2021 [35]	SCI	Mouse	Female	Complete transection at T8/9	Awake cystometry: -Non-voiding contractions were observed at 2 weeks post injury; bladder capacity was increased at 6 weeks; -DSD was observed 2 weeks post injury, but periodic EMG reductions that produce voiding were not observed at this time point, until 4 weeks.	Increase in BDNF at all injury timepoints: higher at 2 weeks and decreases at 4 and 6 weeks, but never returning to basal levels.	Increase in mRNA levels of TRPV1, ASIC1, ASIC3-, and piezo2 DRG L6-S1.	The development of DSD might be related to changes in the expression of mechanosensitive channels such as ASICs and Piezo2; changes in these channels are accompanied by changes in BDNF expression.

Table 1. *Cont.*

Study	Model	Species	Sex	Induction Method	Urodynamic Findings	Changes in Bladder Tissue	Changes in Neuronal Tissue	Therapies/Mechanisms Identified
Oliveira et al., 2019 [26]	SCI	Rat	Female	Complete transection at T8/9	Cystometry under urethane anesthesia: increased frequency of bladder contractions and higher peak pressures; RTX treatment attenuated this.	-Increase in bladder wall thickness; -Increase in bladder TRPV1; RTX treatment attenuated this.	-Severe loss of gray matter arrangement in dorsal and ventral horns, with white-matter disruption and several cavities; -Increase in lumbosacral spinal GAP43 and CGRP expression.	The neurotoxin RTX is capable of reducing urinary symptoms related to SCI; these effects were only seen in the bladder, not affecting the spinal cord.
Ozsoy et al., 2012 [36]	SCI	Rat	Female	Spinal compression at T8	One week after SCI, all groups presented bladder areflexia; in the severe contusion group, urinary function did not improve. Mild contusion rats presented better scores following the third week after lesion.	Detrusor hyperinnervation: increases in β-III-tubulin.		Bladder function was significantly worse following severe compared to moderate compression injury.
Munoz et al., 2017 [37]	SCI	Rat	Female	Spinal contusion at T8/T9: mild and severe	Cystometry under urethane anesthesia: after mild contusion, mice presented increased inter-contractile intervals, high number of non-voiding contractions, and longer duration of the IPHFO (duration of intra-luminal pressure high frequency oscillations). Severe contusion mice showed a high NVC frequency, practically absent IPHFO events, and small voiding volumes.		-GFAP increase (astrogliosis) and microglial activation in both groups 4 weeks after SCI (at injury level).	Independent of the impact intensity, a contusion SCI increases microglia and astrocyte infiltration into the injured region without a strong correlation with locomotor impairment; bladder dysfunction is worse in severe than mild contusion.
Wada et al., 2018 [38]	SCI	Rat	Female	Complete transection at T9–T10	Awake cystometry: the time to the first non-voiding contraction was significantly prolonged after both 0.1 and 1.0 mg/kg of intravenous administration of SC51089; other parameters were not altered.		Increase in PGE2 concentration of the L6 and S1 spinal segments; the mRNA expressions of EP1 receptors of the L6 and S1 spinal cord and DRG from SCI rats were increased.	SC51089-prostaglandin 1 receptor antagonist PGE2-induced EP1 receptor activation is involved in the initiation of C-fiber excitation to trigger NVC in SCI rats.
Wada et al., 2017 [39]	SCI	Rat	Female	Complete transection T8–T9	Awake cystometry: inter-contraction interval, bladder capacity, and bladder compliance were significantly increased in SCI animals treated with combination therapy, and not monotherapies; the time required for the first NVC was significantly prolonged in the oxybutynin and combination group.	Type 3 collagen, HIF-1a, TGF-β1, and FGF-β (actors involved in tissue remodeling and hipoxia) were reduced in oxybutynin and combination therapy; in mirabegron therapy, the expression of mRNA of HIF-1α and TGF-β1 was significantly reduced compared to controls.		The combination therapy of an anticholinergic agent (oxybutynin) and a b3-adrenoceptor agonist (mirabegron) elevated the bladder elastin level, reduced NVCs, and increased bladder compliance to a greater extent than the monotherapy of either drug in SCI.

Table 1. *Cont.*

Study	Model	Species	Sex	Induction Method	Urodynamic Findings	Changes in Bladder Tissue	Changes in Neuronal Tissue	Therapies/Mechanisms Identified
Shneider et al., 2019 [40]	SCI	Rat	Female	Incomplete or complete transection at T8	Awake cystometry: bladder was areflexic the first week after injury; over the following 3 weeks, maximum detrusor pressure constantly increased, exceeding the baseline measurements at 4 weeks; reduction in voiding rates and urine volumes; reduction in bladder compliance; development of DSD. Treatment with aniti-Nogo-A improved several urodynamic parameters.		After 4 weeks, animals treated with vehicles showed decrease in CRF-positive innervation of Lam X; animals treated with anti-Nogo-A antibody presented values similar to intact rats; in the IML region, both injury groups showed a reduced CRF-positive fiber density; anti-Nogo-A antibody-treated rats showed a trend for higher GABAergic values, GAD2 mRNA-positive cells decreased in L6-S1.	Anti-Nogo-A antibody treatment improved urodynamic and electrophysiological parameters in SCI animals, namely a pronounced recovery of the physiological EUS function during voiding. This is likely due to protection of spared descending fibers from the PMC sprouted below the level of the injury in a specific target region, Lam X, thereby restoring functional input from the key bladder control system.
Sadeghmousavi 2022 [41]	SCI	Rabbit	Male	Spinal cord cauterization at T12-L1	Cystometry under urethane anesthesia: increase in bladder capacity, decrease in leak point pressure, increase in leak point volume.	Lymphoid tissue hyperplasia; nerve markers (NF200 and S100) positive at muscular sites.	In injured spinal segments, S100 was increased and NF-200 was diminished.	This method of NLUTD induction was without the leg's neurological deficit, easily applicable, low-cost, and was accompanied by minimal surgical preparation and a satisfactory survival rate in comparison with other SCI animal models.
Horst et al., 2013 [42]	SCI	Rat	Female	Two opposite lateral hemisections (T7 and T11)	Awake cystometry: increased number of non-voiding contractions, showing signs of detrusor overactivity; the treatment significantly attenuated bladder dysfunction, but not to basal levels.	PGP 9.5 (general nerve marker) was increased in trained rats and decreased in non-trained rats (reduced detrusor hypertrophy); NF200 afferent fiber innervation was reduced in non-trained animals; the NF200:PGP ratio was significantly lower in trained RATS; non-trained rats showed a trend for low TH density.		A multisystem neuroprosthetic training program counteracts the emergence of neurogenic bladder dysfunction and improves bladder function in rats with severe SCI.
Frias et al., 2015 [27]	SCI	Rat	Female	Complete transection at T9	Cystometry under urethane anesthesia: one week after SCI, the animals presented bladder areflexia; four-week SCI, NDO was evident, with increase in the values of frequency, peak pressure, baseline pressure, and amplitude of urinary function; chronic BDNF sequestration during the first week promoted an earlier appearance of NDO; BDNF sequestration starting at 4 weeks almost abolished bladder contractions.		Time-dependent increase in BDNF expression: L5–L6 segments; GAP-43 expression increased after SCI in thin fibers coursing in the superficial laminae of the dorsal horn; continuous BDNF sequestration during spinal shock resulted in intense GAP-43 expression; increase in phosphoJNK in laminae I–II of the experimental groups in which axonal sprouting was more pronounced.	BDNF is involved in the long-term maintenance of NDO arising after SCI, as an important modulator of sensory afferent sprouting, the key mechanism underlying NDO emergence.

Table 1. *Cont.*

Study	Model	Species	Sex	Induction Method	Urodynamic Findings	Changes in Bladder Tissue	Changes in Neuronal Tissue	Therapies/Mechanisms Identified
Coelho et al., 2016 [43]	SCI	Rat	Female	Complete transection at T8	Cystometry under urethane anesthesia: increased frequency and basal pressure; decreased amplitude of contractions; treatment with botox normalized these parameters to basal conditions.		Onabot/A cleaves SNAP-25 in L5-S1 spinal segments, coursing laminae I and II of the dorsal horns. Increase in CGRP expression at L5-S1 spinal cord (laminae III and IV) and at DRG level; treatment reduced this. Increase in ATF3 (marker of neuronal stress) expression; treatment further increased this.	Botulinum toxin A improves SCI-induced NDO, acting predominantly on bladder sensory fibres. The mechanism of action of Onabot/A includes the cleavage of SNAP-25 in sensory terminals but also impairment of basic cellular machinery in the cell body of sensory neurons.
Doyle et al., 2018 [44]	SCI	Rat	Male	Complete transection at T8		The level of the muscarinic M2 receptor was decreased in the mucosa of SCI bladders.		Detrusor-mucosa interactions are substantially altered in the neurogenic bladder.
Song et al., 2022 [45]	SCI	Rat	Female	Contusion at T10	Awake cystometry: the basic pressure, maximum pressure, the pressure of leakage point, maximum capacity, residual urine volume, and internal bladder pressure were significantly increased in the SCI group compared with non-SCI groups; treatment improved these features.	-Bladder was enlarged, with a rough and thicker bladder wall; -Abnormal mucosa (both epithelium and lamina propria) with a significant inflammatory infiltrate with neutrophils and monocytes, accompanied by microvascular rupture and bleeding. Treatment improved lamina propria inflammation.	Reduction in mRNAs and proteins of TRPA1, TRPV1, P2X2, and P2X3 both in bladder and L6-S1 DRG; PTNS treatment attenuated this.	Posterior tibial nerve stimulation (PTNS) improves SCI-induced functional and histological impairment of the bladder, via the TRP/P2X signaling pathway.
Wada et al., 2017 [46]	SCI	Mouse	Female	Complete transection at the T8–T9 level	Awake cystometry: bladder capacity, post-void residual urine, and the number of non-voiding contractions during storage were larger when the bladder of SCI animals was only squeezed once daily, compared with twice and thrice.	-At 4 weeks after SCI, the bladder weight was reduced in animals who had their bladders more frequently squeezed; -Levels of NGF protein in the bladder mucosa of SCI mice were higher; -Levels of NGF were lower in animals who had their bladders more frequently squeezed.	The expression of P2X2, P2X3, TRPA1, and TRPV1 mRNA was increased in SCI mice (DRG), when compared to spinal intact mice.	The post-injury bladder management with an increased number of daily bladder emptying improves the storage and voiding LUTD after SCI, associated with the decrease in bladder NGF and reductions in C-fiber afferent marker receptors in bladder afferent pathways.
Sartori et al., 2022 [47]	SCI	Rat	Female	Incomplete transection at the T8–T9 level	Awake cystometry: Increased maximum intravesical pressure, voiding time, post-void residual volume, and the number of non-voiding contractions. Reduced maximum flow rate, voided volume, and voiding efficiency.		Reduction in the density of 5-HT-positive fibers in both lamina X and ventral horn. 5-HT density increased over time, but remains severely affected up to 4 weeks after SCI; -Decrease in CRF-positive fiber density in the intermedio-lateral column (and lamina X), but partially at 4 weeks; -Increase in CGRP density only 2–3 weeks after SCI; -Decrease in the glutamatergic neurons (VGLUT2 mRNA-positive) in the laminae I, II and III of the dorsal horn, but not in laminae IV–V and X; -Decrease in GABAergic cells (GAD2 mRNA-positive) in the laminae I, II, III, IV and V.	Detrusor overactivity is possibly influenced by the sprouting of afferent fibers of type C in the dorsal horn responding to bladder distension, while DSD might be driven by decreased bulbospinal input to and a reduced number of inhibitory GABAergic interneurons in the lumbosacral cord.

Table 1. *Cont.*

Study	Model	Species	Sex	Induction Method	Urodynamic Findings	Changes in Bladder Tissue	Changes in Neuronal Tissue	Therapies/Mechanisms Identified
Elkelini et al., 2012 [48]	SCI	Rat	Female	Complete transection at T4	Awake cystometry: increased number of non-voiding contractions, basal pressure, and increase in maximum voiding pressure; intravesical onabotA reduced the number of uninhibited contractions and lowered the maximum pressure, but had no effect on resting pressure.	Increase in NGF bladder concentration; onabotA treatment significantly reduced NGF levels.	(3 weeks after SCT) Increase in NGF concentration in the dorsal root ganglia (DRG) of the T4 root; onabotA treatment decreased NGF concentration.	Intravesical onabotulinumtoxinA blocks autonomic dysreflexia in rats with T4-SCT, due to a decrease in NGF concentrations at the bladder and dorsal root ganglia T4, which suggests an afferent pathway modulation by intravesical onabotA treatment.
Shimizu et al., 2017 [49]	SCI	Mouse	Female	Complete transection of T8–T9			-Increase in the number of CGRP and TRPV1 promoter vector-labeled cells in L1 and L6 DRG; the median cell size of CGRP neurons was increased, while TRPV1 was decreased; -Decrease in the number of NF200 promoter vector-labeled cells in L6 DRG; the median cell size of the NF200 neurons was larger.	SCI induces morphological changes in bladder afferent pathways, especially in the C-fiber cell population, along with upregulation of CGRP and TRPV1 expression in bladder afferent neurons.
Takahashi et al., 2013 [50]	SCI	Rat	Female	Complete transection at T9–T10			The ratio of Kv1.4 α-subunit staining density in bladder afferent and unlabeled DRG neurons was lower in spinal transected animals.	The excitability of C-fiber bladder afferent neurons is increased in association with reduction in KA current density and Kv1.4 α-subunit expression in SCI rats.
Munoz et al., 2017 [51]	SCI	Rat	Female	Bilateral dorsal lesion T8–T9	Cystometry under urethane anesthesia: SCI increased duration of intraluminal pressure, high-frequency oscillations, and non-voiding contractions' frequency; These parameters were improved by P2X7R antagonist treatment.	-Increased expression of beta-actin marker; -Increased levels of urothelial P2X3 receptors; treatment with P2X7R antagonist attenuated both findings.	-Activation and infiltration of microglia in T7/T8 dorsal horn areas in non-BBG treated SCI groups. -The density of CD11b-positive microglia cells and the percentage of activated microglia were significantly reduced in treated rats.	P2X7R antagonist (BBG) induced a significant reduction in the frequency of non-voiding detrusor contractions, which was correlated with a lower amount of activated microglia.
Sartori et al., 2022 [52]	SCI	Rat	Female	Hemisection at T8–T9	Awake cystometry: transcutaneous tibial nerve stimulation induced fewer episodes of non-voiding contractions, a lower maximum intravesical pressure during the storage phase, a higher voided volume, and a lower post-void residual volume in SCI rats, resulting in a higher voiding efficiency; the beneficial effect in bladder urodynamics disappeared one week after the end of the stimulation period.	The unstimulated sham animals had a bigger and heavier bladder compared with animals that underwent tibial nerve stimulation.	Higher density of CGRP-positive structures in layer I and II of the dorsal horn of L6 and S1 in the stimulated group (not statistically significant).	Application of transcutaneous tibial nerve stimulation in rats early after SCI had a beneficial influence on the development of lower urinary tract dysfunction that typically arises after an incomplete SCI.

Table 1. *Cont.*

Study	Model	Species	Sex	Induction Method	Urodynamic Findings	Changes in Bladder Tissue	Changes in Neuronal Tissue	Therapies/Mechanisms Identified
Cho et al., 2014 [53]	SCI	Rat	Male	T11 damage using a surgical gouge	Cystomery under zolotyl anesthesia: increase in contraction pressure and the contraction time; oral mucosa stem cells transplantation into the injury area ameliorated these features		The transplantation of oral mucosa stem cells decreased the SCI lesion, once new tissues were increased in the surroundings of the damaged tissues, reduced apoptosis, and increased the spinal cord tissues SMA-α and Ki67 expressions; c-Fos and NGF expression in the neuronal voiding centers in SCI animals were also reduced by the treatment.	Transplantation of oral mucosa stem cells ameliorated the SCI-induced neurogenic bladder symptoms by inhibiting apoptosis and enhancing cell proliferation. As result, SCI-induced neuronal activation in the neuronal voiding centers was suppressed, showing the normalization of voiding function.
Yao et al., 2022 [54]	SCI	Rat	Unspecified	Spinal compression at T13	Cystometry under chloral hydrate: placental mesenchymal stem cell-derived neural cell transplantation increased the maximum bladder capacity and bladder compliance, and decreased the bladder basic pressure and urinary leakage pressure in SCI rats.	Placental mesenchymal stem cell-derived neural cell transplantation improves the ultrastructure of detrusor muscle tissue (reduces the rough endoplasmic reticulum, mitochondrial swelling, and thigh fibrils) and improves the elasticity and compliance of detrusor muscles.	-SCI up-regulates caspase-3 protein expression in the spinal cord; the TUNNEL value after placental mesenchymal stem cell transplantation decreased significantly in SCI rats.	Placental mesenchymal stem cell transplantation increased bladder dysfunction after SCI. Cell transplantation can rapidly differentiate into nerve cells to compensate for the massive apoptosis of cells associated with the lesion.
Elkelini et al., 2012 [55]	SCI	Rat	Female	Complete transection at T10	Awake cystometry: transected rats developed uninhibited contractions, increased resting pressure, increased threshold pressure, and increased maximum voiding pressure; short-term sacral neurostimulation reduced the threshold pressure, but no other urodynamic parameters were significantly affected.		Increase in CGRP content (L6DRG); neurostimulation reduced this,	Sacral neuromodulation (SNM) reduced threshold pressure and CGRP content at L6 DRG, which may explain the modulatory effect on the C-fiber afferents supplying the urinary bladder. Chronic stimulation may be required to produce significant changes in all CMG parameters.
Wang et al., 2012 [56]	SCI	Rabbit	Female	Spinal compression by aneurysm clip at T10	Awake cystometry: 2 weeks after SCI, basal pressure, leak-point pressure, and residual urine volume increased; the detrusor was hyperactive during bladder filling, DSD occurred during voiding; bladder compliance was decreased. Four weeks of accumulated sacral anterior root stimulation of anodal block: intravesical pressure, maximum bladder pressure, maximum detrusor pressure, bladder leak-point pressure, resting pressure, and residual volume decreased, while bladder capacity and voiding volume increased.	-Bladder expression of the M2 receptor, P2X3 receptor, and NGF increased in SCI animals; decreased after 4-week electrical stimulation; -Expression of the M3 receptor and β2-adrenergic receptor decreased following SCI, increasing after 4-week electrical stimulation.		Long-term sacral anterior root stimulation of anodal block in rabbits following SCI could repair urinary function. The recovery neurotransmitter receptor expression and decreased NGF expression could be one of the mechanisms of action.

Table 1. *Cont.*

Study	Model	Species	Sex	Induction Method	Urodynamic Findings	Changes in Bladder Tissue	Changes in Neuronal Tissue	Therapies/Mechanisms Identified
Liu et al., 2018 [57]	SCI	Rat	Female	Complete transection at S2	Cystometry under urethane anesthesia: typical voiding contractions of the bladder were not observed in SCI rats, they were replaced by several irregular micturition waves with low amplitude.	-Detrusor hypertrophy; -Increase in mesenchyme matter; -Increase in bladder volume; -The mRNA and protein expression levels of four HCN subtypes were decreased, with the HCN1 channel being the most significant; all four HCN subtypes were expressed in single bladder interstitial cells of Cajal-like cells (ICC-LCs); -The protein levels of Trip8b, Nedd4-2, and NRSF were upregulated, while filamin A was downregulated.		Decreased bladder HCN channel expression and function induced by altered regulatory proteins are involved in the pathological process of SCI-induced neurogenic bladder.
Han et al., 2017 [58]	SCI	Rat	Female	T11 damage using a surgical gouge	Cystometry with zolotyl anesthesia: increased basal contraction pressure and contraction time; tamsulosin treatments decreased the basal contraction pressure and time, dose-dependently.		Increased c-Fos, NGF and NADPH-d expression in the vlPAG, PMC, and spinal dorsal horn (L5); tamsulosin treatment suppressed these increases.	Tamsulosin (α1-adrenoceptor antagonist) treatment suppressed NDO symptoms and c-fos and NGF augmentation after SCI, showing that it can be used to attenuate bladder dysfunction following SCI.
Yang et al., 2017 [59]	SCI	Rat	Female	Contusion at T10	Awake cystometry: reduction in inter-contraction interval, voided volume, and voiding efficiency; increased basal pressure, threshold pressure and bladder capacity. Bladder function was improved by treatment with tanshinone IIAh methylprednisolone.	-Increased bladder weight; -Increase in thickness of bladder detrusor; -Vascular alterations, edema, and proliferation of urothelial layers; the umbrella cell layer was disrupted and a marked neutrophil infiltration to the suburothelial tissue as well as blood vessel congestion and dilation was observed; treatment with tanshinone IIA and methylprednisolone reduced these features.	-Decrease in motor neurons in the anterior horn, paired with a reduction in Nissl body conspicuity; -DRGs L6-S1 presented a large number of inflammatory cells; -DRGs L6-S1 neurons cell bodies became hypertrophic and elongated with some of the nuclei shrunken or disappeared. Some Nissl bodies also disappeared or were replaced by vacuoles. All these features were attenuated by Tanshinone IIA and methylprednisolone treatments.	Tanshinone IIA and methylprednisolone improved functional recovery after SCI-induced lower urinary tract dysfunction by remodeling the spinal pathway involved in lower urinary tract control.
Lee at al., 2012 [60]	SCI	Rat	Female	Contusion at T8		-Increased bladder weight; -Increased mRNA expression of GAPDH. In the control group, the most-expressed α1 AR subtype was α1a and in the SCI group α1d, but SCI had lower expression than all the suited receptors (α1a, α1b, α1d).		SCI modulates the α1 AR mRNA subtypes in rat urinary bladder. The relatively increased α1d or decreased α1a AR mRNA expression may be a therapeutic candidate for controlling symptoms of neurogenic bladder after SCI.
Cho et al., 2020 [61]	SCI	Rat	Male	Contusion at T8	Awake cystometry: decreased bladder contraction pressure and contraction time; increased intercontractional interval. TGN administration attenuated these changes.		Increased VEGF, NGF, and BDNF expression in spinal injury site; TGN treatment suppressed the expression.	Treatment with a PTEN inhibitor (TGN) induced functional recovery and resulted in significantly lower expression of VEGF, NGF, and BDNF at injury site.

Table 1. *Cont.*

Study	Model	Species	Sex	Induction Method	Urodynamic Findings	Changes in Bladder Tissue	Changes in Neuronal Tissue	Therapies/Mechanisms Identified
Cui et al., 2021 [62]	SCI	Rat	Male	Complete transection at T9		-Increase in collagen and reduction in smooth muscle fibers; disorganization of these fibers' distribution; -The ratio of type I/III collagen in bladder smooth muscle cells was higher than in controls. Treatment with 3-methyladenine improved the overall histological changes.	-Enlargement of the space around the nerve cells in the spinal cord; appearance of blurred nucleolus, swollen cells, and vaculose. After treatment, the number of necrotic nerve cells and vacuoles in the spinal cord tissue was reduced and the degree of inflammatory infiltration was reduced; -Increased LC3-II expression levels; treatment reduced them; -Reduced MBP expression; treatment increased them.	3-methyladenine reduces the loss of MBP and inhibits bladder detrusor dysfunction by inhibiting the autophagy response in bladder detrusor muscle cells. The inhibition of collagen fiber expression in the detrusor promotes the recovery of bladder function.
Shimizu et al., 2021 [63]	SCI	Mouse	Female	Complete transection at T8–T9	Awake cystometry: the number of NVCs was significantly reduced in vibegron-treated SCI mice compared to vehicle-treated control SCI mice.		Increased mRNA levels of TRPV1, TRPA1, ATF3, and iNOS in L6-S1 DRG were increased in SCI mice versus SI mice and decreased after vibegron treatment.	β3-adrenoceptor activation by vibegron improved the SCI-induced storage dysfunction, possibly through the reduction in C-fiber-related receptor expression and inflammation-related markers in DRG.
Gil-Tomee et al., 2019 [64]	PD	Mouse	Both	GM2 synthase knockout (KO) mice	KO mice initially had more void spots that reduced with age. KO mice initially had bladder hyperreflexia and then developed hyporeflexia.	-Increased proNGF protein levels; -Abnormal myelination in bladder nerves.	Loss of TH in the VTA of GM2 KO mice compared to WT mice.	Dopaminergic damage in brain micturition centers impact voiding in association with a loss in mature ganglioside.
McMillan et al., 2014 [65]	MS	Mouse	Male	Experimental autoimmune encephalomyelitis (EAE)	Voiding spot assay: increased number of non-voiding contractions; decreased voided volume; shortened intermicturition intervals; decreased bladder capacity; reduced volume of voided urine.		-Upregulation of GFAP expression (gliosis) and decrease in MBP at the lesion site; -Several proinflammatory cytokines were upregulated in the brains at 1 wk, followed by a gradual recovery to baseline values by 4 wk -In the spinal cord, only IFN-γ levels were upregulated at 1 wk, suggestive of a mild inflammatory reaction; normal levels were observed at 4 wk.	Coronavirus-induced demyelination of the CNS causes the development of a neurogenic bladder that is comparable with neurogenic detrusor overactivity observed in patients with multiple sclerosis.
Altuntas et al., 2012 [66]	MS	Mouse	Female	Experimental autoimmune encephalomyelitis (EAE)		-Increase in collagen; -Decrease in NGF, GDNF, muscarinic receptors, and purinergic receptors; -Increased expression of CTGF and TGF-β3 mRNAs (markers of the fibrosis cascade),		EAE-caused neurological disability in mice and contributes to marked bladder remodeling. Although all three components of detrusor smooth muscle, urothelium, and connective tissue contribute to the increased bladder mass, the role of connective tissue is more prominent and potentially detrimental.

Table 1. *Cont.*

Study	Model	Species	Sex	Induction Method	Urodynamic Findings	Changes in Bladder Tissue	Changes in Neuronal Tissue	Therapies/Mechanisms Identified
Lee et al., 2019 [67]	MS	Mouse	Male	Coronavirus-induced encephalitis (CIE)	Voiding spot assay: at 10 wks post-inoculation, bladder capacity, the inter-micturition interval, and bladder pressure at voiding in all groups, except for the C-RELAP group, were similar to the respective values in the control group. Mice in the C-RELAP group developed overactive bladder phenotype. This means that the C-RELAP group develop a more severe and long-lasting type of neurogenic bladder overactivity than other groups, providing evidence of some correlation between the type of neurodegenerative changes in the CNS and type of developed voiding dysfunction in CIE mice.	Increased expression of TNF-α, Increased content of IFN-γ, IL-2, TGF-β and TNF-α	Decreased expression of IL-1β and IL-10 in the brain. The C-PRO group was characterized by a decreased expression of IL-1β, IL-6, IL-10, IL-17, and TNF-α. C-RELAP mice had a significantly reduced level of IL-4 in the brain.	Mice with CIE developed three phenotypes of neurologic impairment, mimicking different types of MS.
Jin et al., 2017 [68]	MS	Mouse	Unspecified	Experimental autoimmune encephalomyelitis (EAE)	Voiding spot assay: decrease in urine volume voided per micturition and increased frequency of urination; increased bladder diameter; features reverted with treatment.	-Decreased number of ICCs (c-Kit staining); treatment partially reverted this; -Increased expression of Panx1 and Cx43.		The effect of SCF on the loss of ICCs may offer a theoretical option for treating patients with MS-related voiding dysfunction.
Xue et al., 2013 [69]	MS	Mouse	Female	Experimental autoimmune encephalomyelitis (EAE)	-EP3 and EP4 agonists increased micturition frequency and decreased void weight in EAE mice, compared with control mice treated with vehicles.	-The concentration of PGE2 level in the bladder increased as the EAE (severity) score increased; -Bladder weight to total body weight ratio was higher; -The expression of EP3 and EP4 receptors increased as EAE severity score increased, but no change in expression of EP1 and EP2 receptors was verified.		EAE-induced upregulation of EP3 and EP4 receptors in the bladder was accompanied by bladder dysfunction. However, EAE had no significant effect on EP1 and EP2 receptors.
Liang et al., 2016 [70]	Cerebral ischemia	Rat	Female	Middle cerebral artery occlusion (MCAO)	-Increased peak voiding pressure and residual volume at 1 day, 3 days, and 7 days following ischemia induction, which declined after pre- and postischemic CD34+ cells treatment; -Decreased voided volumes and intercontraction intervals decreased after ischemia induction, but increased after pre- and postischemic CD34+ cell treatment at 3 days and 7 days.	NGF, M2, and M3 mRNA expression and immunoreactivity (except for M2) decreased after MCAO, but increased after preischemic CD34+ cell treatment.		Bladder dysfunction caused by MCAO may have a normal micturition restored by treatment with human umbilical cord blood-derived CD34+ cells. This urinary function improvement may be related to the expression of bladder NGF, M2, and M3.

Table 1. *Cont.*

Study	Model	Species	Sex	Induction Method	Urodynamic Findings	Changes in Bladder Tissue	Changes in Neuronal Tissue	Therapies/Mechanisms Identified
Campeau et al., 2014 [71]	PD	Rat	Female	Injection of 6-hydroxydopamine in the right medial forebrain bundle	Awake cystometry: increased bladder capacity, post-void volume, and threshold pressure; decreased after treatment with rBMSCs; urodynamic effects of the 6-OHDA lesion up to 42 days after injection.		-GFAP expression was present around rBMSC and ErBMSC graft sites, unlike the saline injection site, suggesting activated astrocytes around the graft sites; -There was IBA-1 expression at the injection site in all groups, but it vanished by the fourth week. Microglia infiltration was present around injected rBMSCs and ErBMSCs.	Transplantation of rBMSCs alone improved urodynamic pressure at 42 days after treatment more markedly than ErBMSCs. This improvement in rBMSC rats was associated with a higher number of TH-positive neurons in the treated SNpc, suggesting that functional improvement may require a juxtacrine effect.
Cho et al., 2015 [72]	Intracerebral hemorrhage (ICH)	Rat	Female	Induction of ICH in the hippocampal CA1 region was performed using a stereotaxic frame and collagenase	Cystometry under zolotyl anesthesia: bladder contraction pressure and time were significantly increased and the voiding pressure and time decreased by the induction of ICH, as compared with the control treatment.		-c-Fos expression levels in the neuronal voiding centers (medial preoptic area, ventrolateral gray, pontine micturition center, and SC L4-L5) were increased; -NGF expression levels in the neuronal voiding centers were increased.	ICH-induced NLUTD rat model may be a more appropriate method to analyze NLUTD in stroke patients than a cerebral infarction model.
Pritchard et al., 2017 [73]	PD	Marmoset	Both	MPTP injection			-Reduction in tyrosine hydroxylase expression in the substantia nigra.	The increased neurogenically mediated contractions where no extrinsic innervation exists might be due to long-term adaptive changes locally as a result of the loss of the nigrostriatal output.
Shen et al., 2013 [74]	Meningomyelocele	Rat	Fetuses from female rats	Gavage feeding of retinoic acid at embryonic day 10 (E10)		Decrease in β-III-Ttubulin-content at E16, E18, and E20.	-Increase in GFAP expression (in the dorsal region of the spinal cord); -Decrease in VAChT expression in the dorsal lateral nucleus of the spinal cord at all fetal ages.	Smooth muscle of the bladder in fetal rats with myelomeningocele is morphologically normal, while the innervation of the smooth muscle of the bladder is markedly decreased centrally and peripherally. Astrocytosis appears in a later embryonic stage, which could be related to nerve repair in the spinal cord.
Tekin et al., 2016 [75]	Myelomeningocele	Rat	Fetuses from pregnant female rats	Gavage feeding of retinoic acid at embryonic day 10 (E10)			-The interstitial cells of Cajal (ICC) score of the MMC group is decreased.	The density of the ICC in the urinary bladder decreased in the neurogenic bladder developed in MMC.

Table 1. *Cont.*

Study	Model	Species	Sex	Induction Method	Urodynamic Findings	Changes in Bladder Tissue	Changes in Neuronal Tissue	Therapies/Mechanisms Identified
Liu et al., 2022 [76]	Myelomeningocele	Rat	Fetuses from pregnant rats	Gavage feeding of retinoic acid at embryonic day 10 (E10)		-Inhibition of bladder cells proliferation, due to increased apoptosis in late embryonic stage (increased cleaved caspase 3); -Increase in α-SMA mRNA; -NeuN protein expression increased with time, with no significant difference between the MMC and CRL groups from E16 to E18; however, the expression of NeuN protein was significantly lower in the MMC group than in the CRL group from E20 to E22.		Bladder dysfunction in myelomeningocele fetal rats is related to the inhibition of proliferation, promotion of apoptosis, and reduction in bladder nerve and smooth muscle-related protein synthesis.

2.1. Induction Model and Assessment of Bladder Function

LUT dysfunction is a common consequence of several neurologic diseases. The level at which they occur may provoke distinct urinary complications (Figure 2A), such as NDO [8]. The vast majority of the analyzed reports, 74%, use spinal cord injury as an NDO model (Figure 2B). From these publications, 59% report complete transection of thoracic segments to induce SCI [26–35,38,39,43,44,46,48–50,55,57,62,63], followed by spinal contusion (13%). The use of spinal hemisections was less frequent (10%) [42,47,52], as were spinal compressions (8%) [32,36,54,56] and other SCI methods (10%) [41,51,53,58] (Figure 3). Regarding the SCI model of NDO, we found that, irrespective of the type of injury, the thoracic level was the preferred level to inflict spinal lesion, with the T8–T9 levels being particularly favored. Only one study used a lesion at a higher level (T4) [48] (Figure 3).

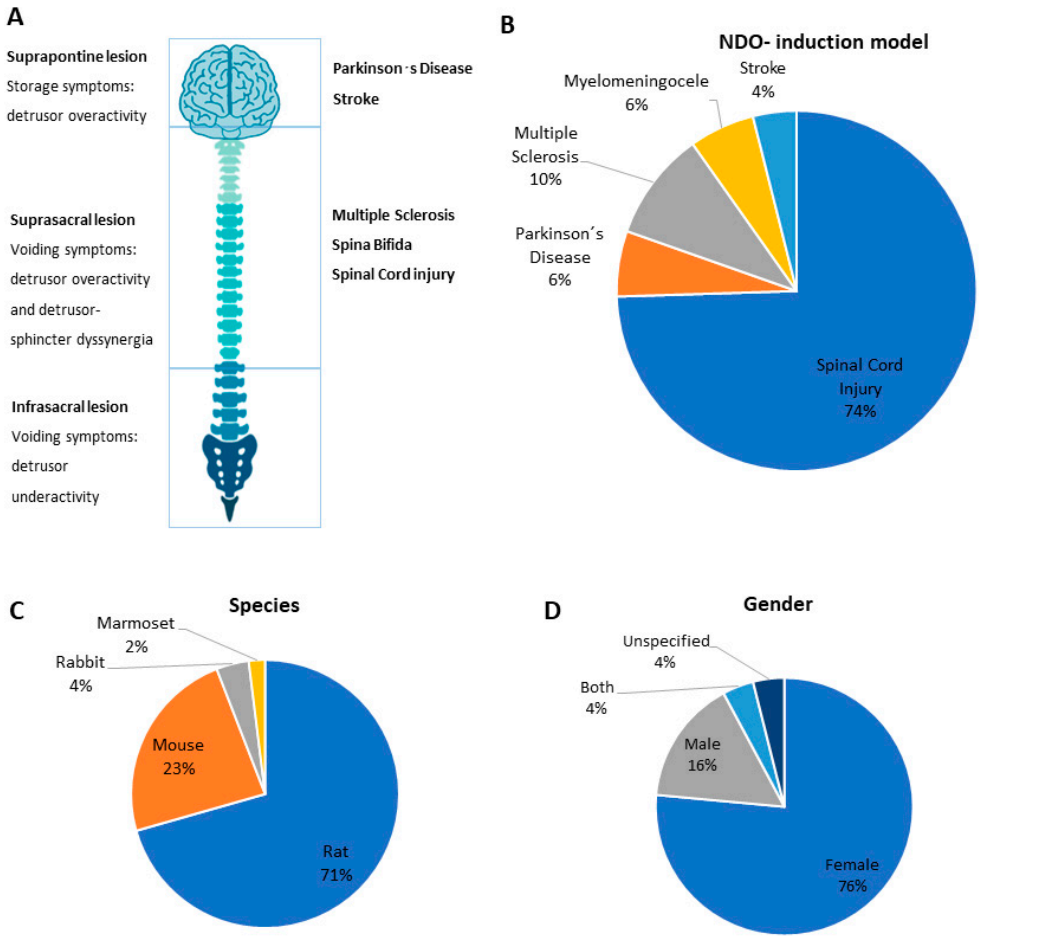

Figure 2. NDO-driven pathologies and animal models to induce them. (**A**) The consequences of central nervous system (CNS) injuries for urinary function are influenced by the level on which they occur: supraspontine lesions are associated with storage symptoms, suprasacral lesions are characterized by voiding symptoms, namely detrusor overactivity and detrusor sphincter dyssynergia, and infrasacral lesions are often associated with detrusor underactivity. (**B**) Animal models to induce neurogenic detrusor overactivity (NDO) are based on supraspontine or suprasacral lesions and include animal models of spinal cord injury (SCI), Parkinson's disease (PD), multiple sclerosis (MS), myelomeningocele, and stroke. (**C**) The species used to induce NDO models are predominantly rats and mice, with little use of other small mammals, such as rabbits and marmosets. (**D**) Female animals were predominantly used.

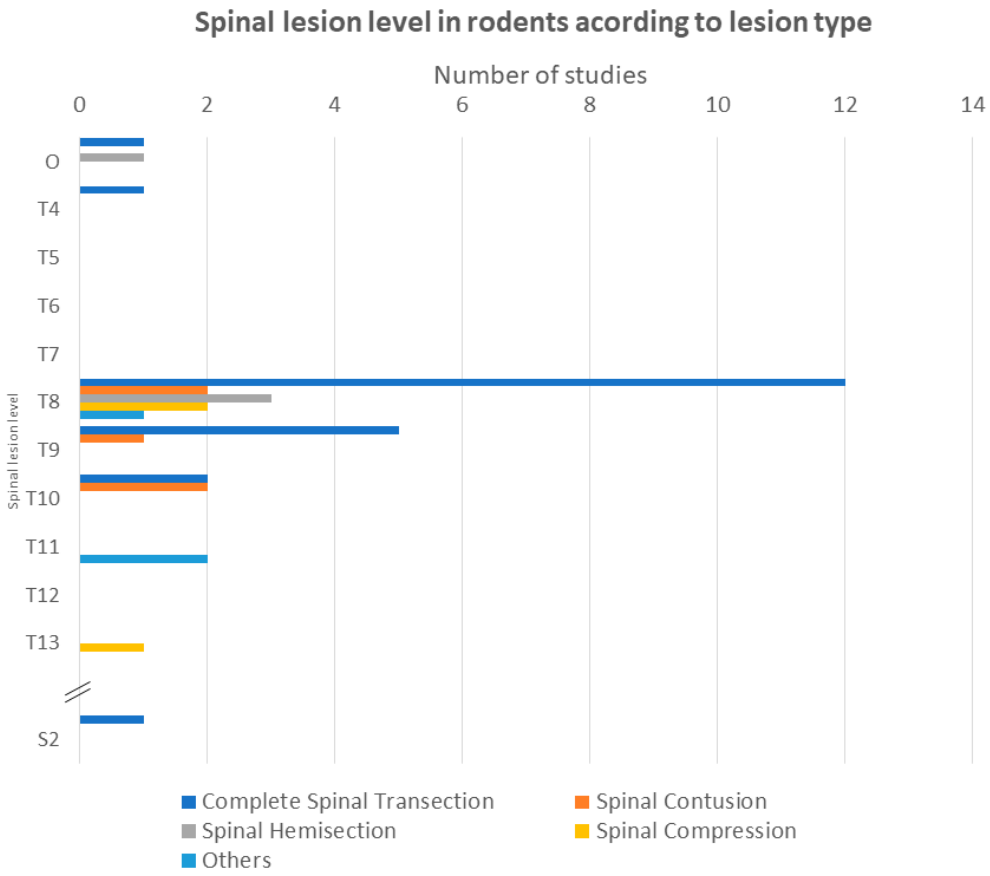

Figure 3. Injured spinal cord levels according to lesion type. O: Other—this category includes one publication which did not specify the spinal lesion level and another study that reported a complex spinal cord injury (involving non-contiguous spinal segments). Since many studies present their spinal lesion level as a combined lesion of two contiguous segments (e.g., T8–T9), we only considered the upper segment in order to graphically represent these data. This graph only represents data reported from rodents (rats and mice) due to their similar vertebral formula. Only two studies used non-rodent animals to induce SCI, and both relied on lagomorphs (rabbits)—which have a distinct vertebral formula.

The second most-used animal models to produce NDO were related to neurodegenerative disorders. MS was reported in 10% of the articles reviewed and it was induced by promoting experimental autoimmune encephalomyelitis (EAE) [66,68,69] and coronavirus-induced encephalitis (CIE) [65,67]. PD was reproduced in 6% of the studies, using pharmacological induction [71,73] or genetic models [64]. Animal models associated with meningomyelocele (6%), which used retinoic acid as the induction model, stroke (4%), using middle cerebral artery occlusion (MCAO) [70] to resemble cerebral ischemia, and cerebral hemorrhage in the hippocampus to reproduce hemorrhagic stroke [72] were less frequent.

The vast majority of selected studies (71%) performed urodynamic evaluation of the animals to confirm the presence of NDO. Signs of LUT dysfunction were evident, with animals presenting NDO characteristic features: increased micturition frequency; increased number of non-voiding contractions, basal pressure, maximum voiding pressure, and threshold pressure; and high residual volumes. Moreover, decreased voiding volume, maximum flow rate, and voiding efficiency were also referred to. In cases of traumatic models (such as SCI), the setting of the NDO phenotype was preceded by a period of neurogenic shock, with little or no bladder activity, that lasted up to 14 days post-injury. In

the remaining pathologies (MS, PD, stroke), NDO symptoms were present immediately after model induction.

Most of the studies reporting urodynamic recordings used awake cystometry as the recording method (37%) [31–35,38,39,42,46–48,52,56,59,63,68,70,71]. Cystometry under anesthesia represented 26% of studies, with 16% using urethane delivered via subcutaneous [26–28,37,41,43,51] or intraperitoneal injection [57], 8% using zolotyl [53,58,61,74], and 2% referring the use of chloral hydrate as anesthetic [54]. Finally, 8% of the studies used non-invasive voiding spot assays to evaluate bladder function [64,65,67,68]. Surprisingly, 29% of the studies did not describe any assessment of lower urinary tract function [29,30,36,44,45,49,50,60,62,66,69,73–76]. More data on changes in bladder function can be found in Table 1.

2.2. Animal Species and Sex

Concerning the animal species used in NDO models, rodents were preferred, with 71% of studies using rats. Other animals were less frequently used and included mice (23%), rabbits (4%), and non-human primates (marmosets) (2%) (Figure 1C). The majority of studies used female animals (76%). Males were used in 16% of selected studies, while only 3% used both male and female animals. Curiously, 5% of studies did not specify which sex was used (Figure 1D).

2.3. Changes in Bladder Tissue

Many studies presented significant findings regarding gross tissue and cellular morphology of the bladder. Animals with an NDO phenotype presented larger bladder volumes and weights than control animals. Bladder tissue was also more fibrotic in NDO animals, associated with detrusor hypertrophy, features which led to a bladder wall thickness increase. The urothelial layer was usually damaged and disorganized, and inflammatory features were conspicuous in the NDO phenotype—leucocyte infiltration in the lamina propria, lymphoid tissue hypertrophy, and vascular congestion and rupture were described. Regarding the ultrastructure of the detrusor smooth muscle cells, some ultrastructural changes were described following NDO induction, such as mitochondrial swelling and rough endoplasmic reticulum hypertrophy.

Several molecular factors were found to play a crucial role in the genesis of NDO and their expression depends on the NDO model and on the histological layer of the bladder wall being analyzed. It is possible to find the most relevant molecular factors explored in the included studies in Table 2 (changes in the bladder) and Table 3 (changes in neuronal tissues), along with the treatments that reportedly reverted or attenuated the NDO-related expression change. In summary, neurotrophic factors are overexpressed in the bladder of chronic SCI and PD animals and underexpressed in the bladder of MS and stroke animals. Inflammatory markers, apoptosis-related factors, and ischemia- and fibrosis-related molecules are upregulated in the bladder tissue of animals with NDO, irrespective of the NDO animal model. Purinergic, cholinergic, and adrenergic receptors are downregulated, although there are some contradictory results, as well as neuronal markers.

2.4. Changes in Neuronal Tissue

Several reports indicate the occurrence of multiple changes in neuronal tissue of NDO animals, largely depending on the NDO model. As SCI was the most commonly used NDO model, the following refers to SCI, unless otherwise indicated. Following SCI, microglial and astrocyte activation was evident, along with the establishment of a pro-fibrotic scar tissue at the spinal lesion site. Gray and white matter disorganization was also reported, with neuronal cells number and their Nissl bodies being reduced. Various inflammatory cells infiltrated the lesion level. In studies using a brain injection of an active substance to induce NDO, the lesion site was reported to show gliosis and inflammatory infiltration, similar to what had been observed in SCI animals.

Table 2. Expression of molecular markers in the bladder wall after NDO induction according to NDO model and tissue layer. Every molecule with a statistically significant expression variation ($p < 0.05$) in the included papers is present in this table. Molecules are split into categories and ordered alphabetically. BDNF: brain-derived neurotrophic factor, GDNF: glial cell line-derived neurotrophic factor, NGF: nerve growth factor, IFN-γ: interferon-gamma, IL: interleukin, TNF-α: tumor necrosis factor alpha, GAPDH: glyceraldehyde 3-phosphate dehydrogenase, EP: prostaglandin E_2 receptor, pTrkA: phosphorylated tropomyosin receptor kinase A, HCN channels: hyperpolarization-activated cyclic nucleotide-gated channel, Panx 1: pannexin 1, TRPA-1: transient receptor potential ankyrin-1, TRPM-4: transient receptor potential melastin-4, TRPV-1: transient receptor potential vanilloid-1, NF200: neurofilament 200, NPY: neuropeptide Y, PGP 9.5: protein gene product 9.5, SYP: synaptophysin, CTGF: connective tissue growth factor, FGF: fibroblast growth factor, HIF-1α: hypoxia-inducible factor 1-alpha, TGF: transforming growth factor, VEGF-α: vascular endothelial growth factor-alpha, Cx 43: connexin 43, Nedd4-2: neural precursor-expressed developmentally down-regulated protein 4-2, NRSF: neuron-restrictive silencer factor, pAkt: phosphorylated Ak strain transforming, Trip8b: tetratricopeptide-repeat containing Rab8b-interacting protein. ↑: expression increase, ↓: expression decrease, †: expression variation detected through protein analysis, ‡: expression variation detected through RNA analysis, CI: cerebral infarction, MMC: myelomeningocele, MS: multiple sclerosis, PD: Parkinson's disease, SCI [a]: acute spinal cord injury (defined here by ≤14 days from NDO induction), SCI [c]: chronic spinal cord injury (defined here by >14 days from NDO induction), M: mucosa, D: detrusor (either detrusor smooth muscle or detrusor stroma), W: whole bladder, E20–22: expression variation only detected on embryonic day 20–22, *: return to basal between 1 and 2 months after NDO induction.

Molecular Factors	NDO Model	Tissue Layer	Bladder Expression after NDO Induction	Treatments That Reverted or Attenuated NDO-Related Expression Change
Neurotrophic factors				
BDNF † [35]	SCI [a,c]	M	↑	
NGF †‡ [29,46,48,56]	SCI [c]	W †‡	↑	siRNA Intravesical onabotulinumtoxinA
		M †	↑	Increased number of daily bladder emptyings
		D †	↑	Long-term sacral anterior root stimulation
Apoptosis-related factors				
GAPDH ‡ [60]	SCI [c]	W	↑	
Receptors				
M2 receptor †‡ [44,56]	SCI [c]	M †‡	↓	
		D †	↑	Long-term sacral anterior root stimulation (detrusor stroma)
M3 receptor †‡ [33,44,56]	SCI [c]	M ‡	↓	
		D †	↓	Long-term sacral anterior root stimulation (detrusor stroma)
		W ‡	↓	M3 RNAi lentivirus
pTrkA † [29]	SCI [c]	W	↑	
P2X2 †‡ [45]	SCI [c]	W	↓	Posterior tibial nerve stimulation (PTNS)
P2X3 †‡ [45,51,56]	SCI [c]	W †‡	↓	Posterior tibial nerve stimulation (PTNS)
		M†	↑	BBG (P2X7R antagonist) Long-term sacral anterior root stimulation
α1a, α1b, α1d -adrenergic receptors ‡ [60]	SCI [c]	W	↓	
β2-adrenergic receptors † [56]	SCI [c]	M	↓	Long-term sacral anterior root stimulation
Ionic channel proteins				
HCN channel proteins †‡ [57]	SCI [c]	W	↓	
TRPA-1 †‡ [45]	SCI [c]	W	↓	Posterior tibial nerve stimulation (PTNS)
TRPM-4 † [30]	SCI [a]	M, D	↑*	9-phenanthrol (TRPM-4 antagonist)
TRPV-1 †‡ [26,32,45]	SCI [c]	W	↑†[c] ↓†‡[c]	Inosine (decreases) Neurotoxin RTX (decreases) Posterior tibial nerve stimulation (PTNS) (increases)
Neuronal markers				
NF200 † [32,41,42]	SCI [c]	W	↓	Inosine Multisystem neuroprosthetic training program
		D	↓	
NPY † [42]	SCI [c]	W	↓	Multisystem neuroprosthetic training program
PGP 9.5 † [42]	SCI [c]	W	↓	Multisystem neuroprosthetic training program
SYP † [32]	SCI [c]	W	↓	Inosine
β-III-tubulin † [36]	SCI [c]	W	↑	

Table 2. *Cont.*

Molecular Factors	NDO Model	Tissue Layer	Bladder Expression after NDO Induction	Treatments That Reverted or Attenuated NDO-Related Expression Change
Ischemia- and fibrosis-related molecules				
FGF-2 ‡ [31]	SCI [c]	W	↑	
FGF-β ‡ [39]	SCI [c]	W	↑	Oxybutynin (anti-cholinergic agent)
HIF-1α ‡ [31,39]	SCI [c]	W	↑	Vibegron and mirabegron (β3-adrenoceptor agonists) Oxybutynin (anti-cholinergic agent)
TGF-β1 †‡ [31,34,39]	SCI [c]	W	↑	Vibegron and mirabegron (β3-adrenoceptor agonists) Oxybutynin (anti-cholinergic agent) BoNTA injection
VEGF-α ‡ [31]	SCI [c]	W	↑	
Other molecules				
Filamin A † [57]	SCI [c]	W	↓	
Nedd4-2 † [57]	SCI [c]	W	↑	
NRSF † [57]	SCI [c]	W	↑	
pAKT † [29]	SCI [c]	W	↑	
Trip8b † [57]	SCI [c]	W	↑	
Molecular Factors	**NDO Model**	**Site**	**Bladder Expression after NDO Induction**	**Treatments That Reverted or Attenuated NDO-Related Expression Change**
Neurotrophic factors				
GDNF ‡ [66]	MS	W	↓	
NGF ‡ [66]	MS	W	↓	
Inflammatory mediators				
IFN-γ † [67]	MS	W	↑	
IL-2 † [67]	MS	W	↑	
TNF-α † [67]	MS	W	↑	
Receptors				
c-kit † [68]	MS	W	↓	Stem cell factor cytokine injection
EP-3 and EP-4 † [69]	MS	W	↑	
M3 receptor ‡ [66]	MS	W	↓	
P2X1 ‡ [66]	MS	W	↓	
Ionic channel proteins				
Panx 1 † [68]	MS	W	↑	Stem cell factor cytokine injection
Ischemia- and fibrosis-related molecules				
CTGF ‡ [66]	MS	W	↑	
TGF-β1 † [67]	MS	W	↑	
TGF-β3 ‡ [66]	MS	W	↑	
Other molecules				
Cx 43 † [68]	MS	W	↑	Stem cell factor cytokine injection
Molecular Factors	**NDO Model**	**Site**	**Bladder Expression after NDO Induction**	**Treatments That Reverted or Attenuated NDO-Related Expression Change**
Neurotrophic factors				
NGF †‡ [70]	CI	W	↓	Treatment with human umbilical cord blood-derived CD34+ cells
Receptors				
M2 receptor †‡ [70]	CI	W	↓‡ ↑†	Treatment with human umbilical cord blood-derived CD34+ cells
M3 receptor †‡ [70]	CI	W	↓	Treatment with human umbilical cord blood-derived CD34+ cells
Molecular Factors	**NDO Model**	**Site**	**Bladder Expression after NDO Induction**	**Treatments That Reverted or Attenuated NDO-Related Expression Change**
Neurotrophic factors				
Pro-NGF † [64]	PD	W	↑	
Molecular Factors	**NDO Model**	**Site**	**Bladder Expression after NDO Induction**	**Treatments That Reverted or Attenuated NDO-Related Expression Change**
Apoptosis-related factors				
Caspase-3 † [76]	MMC	M, D	↑	
Neuronal markers				
NeuN † [76]	MMC	W (E20–22)	↓	
β-III-tubulin † [74]	MMC	D	↓	

Table 3. Expression of molecular markers in the nervous system (NS) after NDO induction according to NDO model and NS site. Every molecule with a statistically significant expression variation ($p < 0.05$) in the included papers is present in this table. Molecules are split into categories and ordered alphabetically. BDNF: brain-derived neurotrophic factor, GAP-43: growth-associated protein 43, NGF: nerve growth factor, IFN-γ: interferon-gamma, IL: interleukin, PGE2: prostaglandin E_2, TNF-α: tumor necrosis factor alpha, CSPG-R: chondroitin sulfate proteoglycans receptors, EP: prostaglandin E_2 receptor, MAI-R: myelin-associated inhibitors receptors, ASIC: acid-sensing ion channel, Kv1.4 α-subunit: voltage-activated Shaker-related potassium channel 1.4 alpha-subunit, TRPA-1: transient receptor potential ankyrin-1, TRPV-1: transient receptor potential vanilloid-1, 5-HT: 5-hydrxytryptamine, CGRP: calcitonin gene-related peptide, CRF: corticotropic-releasing factor, GAD2: glutamic acid decarboxylase, NF200: neurofilament 200, TH: tyrosine hydroxylase, VAChT: vesicular acetylcholine transporter, VGLUT-2: vesicular glutamate transporter 2, VEGF-α: vascular endothelial growth factor-alpha, MAG: myelin-associated glycoprotein, MBP: myelin basic protein, OMgp: oligodendrocyte-myelin glycoprotein, RGMa: repulsive guidance molecule A, ATF-3: cyclic AMP-dependent transcription factor-3, GFAP: glial fibrillary acidic protein, iNOS: inducible nitric oxide synthase, LC3-II: microtubule-associated protein 1A/1B-light chain 3-phosphatidylethanolamine conjugate, NADPH-d: dihydronicotinamide adenine dinucleotide phosphate diaphorase, pJNK: phosphorylated c-Jun N-terminal kinases. ↑: expression increase, ↓: expression decrease, †: expression variation detected through protein analysis, ‡: expression variation detected through RNA analysis, •: expression variation detected through number of cells expressing the studied protein. CI: cerebral ischemia, DRG: dorsal root ganglia, ICH: intracerebral hemorrhage, MMC: myelomeningocele, MS: multiple sclerosis, PD: Parkinson's disease, SC: spinal cord, SCI [a]: scute spinal cord injury (defined here by ≤14 days from NDO induction), SCI [c]: chronic spinal cord injury (defined here by >14 days from NDO induction), only E20–22: expression variation only detected in embryonic days 20 to 22, *: return to basal between 1–2 months after NDO induction, 1 w: 1 week after NDO induction, 10 w: 10 weeks after NDO induction.

Molecular Factors	NDO Model	Location within the Nervous System	Expression after NDO Induction	Treatments That Reverted or Attenuated NDO-Related Expression Change
Neurotrophic factors				
BDNF † [27,61]	SCI [a,c]	Lumbar spinal cord [a,c]	↑	
		Spinal injury level [c]	↑	Treatment with a PTEN inhibitor (TGN)
NGF †• [48,53,57,61]	SCI [c]	T4 DRG †	↑	
		Neuronal voiding centers •	↑	Transplantation of oral mucosa stem cells Tamsolusin
		Spinal injury site †	↑	Treatment with a PTEN inhibitor (TGN)
Inflammatory mediators				
PGE2 † [38]	SCI [c]	Lumbosacral spinal cord	↑	
Apoptosis-related factors				
Caspase-3 † [54]	SCI [c]	Spinal injury level	↑	Placental mesenchymal stem cell transplantation
Receptors				
CSPG-R ‡ [28]	SCI [c]	L5-S1 DRG	↓	
EP-1 ‡ [38]	SCI [c]	Lumbosacral spinal cord	↑	
MAI-R ‡ [28]	SCI [c]	L5-S1 DRG	↓	
P2X2 †‡ [45,46]	SCI [c]	L6-S1 DRG	↑ ‡ ↓ †‡	Increased number of daily bladder emptyings (decreases) Posterior tibial nerve stimulation (PTNS) (increases)
P2X3 †‡ [45,46]	SCI [c]	L6-S1 DRG	↑ [c] ‡ ↓ [c] †‡	Increased number of daily bladder emptyings (decreases) Posterior tibial nerve stimulation (PTNS) (increases)
Ionic channel proteins				
ASIC1 ‡ [35]	SCI [a,c]	L6-S1 DRG	↑	
ASIC2 ‡ [35]	SCI [a,c]	L6-S1 DRG	↑ *	
ASIC3 ‡ [35]	SCI [c]	L6-S1 DRG	↑ *	
Kv1.4 α-subunit ‡ [50]	SCI [c]	L6 DRG bladder afferent neurons	↓	
Piezo2 ‡ [35]	SCI [c]	L6-S1 DRG	↑	
TRPA-1 †‡ [45,46,63]	SCI [c]	L6-S1 DRGs	↑ [c] ‡ ↓ [c] †‡	Increased number of daily bladder emptyings (decreases) Vibegron (decreases) Posterior tibial nerve stimulation (PTNS) (increases)

Table 3. *Cont.*

Molecular Factors	NDO Model	Location within the Nervous System	Expression after NDO Induction	Treatments That Reverted or Attenuated NDO-Related Expression Change
TRPV-1 †‡• [35,45,46,49,63]	SCI [a,c]	L1, L6-S1 DRG †‡• [a,c]	↑ [a,c] ‡• ↓ [c] †‡	Increased number of daily bladder emptyings (decreases) Vibegron (decreases) Posterior tibial nerve stimulation (PTNS) (increases)
		L6 DRG bladder afferent neurons [c] ‡	↑	
Neuronal markers				
5-HT † [47]	SCI [a,c]	Lumbosacral spinal cord (lamina X and ventral horn)	↓	
CGRP †‡• [26,43,47,49]	SCI [c]	Lumbosacral spinal cord (Dorsal horn) †	↑	
		L1 and L6 DRG •	↑	Sacral neuromodulation
		L6 DRG bladder afferent neurons ‡	↑	
CRF † [40,47]	SCI [a,c]	Lumbosacral spinal cord (lamina X and intermediolateral column)	↓	Anti-Nogo-A antibody
GAD2 †‡ [40,47]	SCI [c]	Lumbosacral spinal cord	↓	Anti-Nogo-A antibody
NF200 • [49]	SCI [c]	L6 DRG	↓	
VGLUT-2 ‡ [47]	SCI [c]	Lumbosacral spinal cord (laminae I-III)	↓	
Ischemia and fibrosis-related molecules				
Neurocan † [28]	SCI [a]	Lumbosacral spinal cord	↑ *	
Phosphacan † [28]	SCI [a]	Lumbosacral spinal cord	↑ *	
VEGF-α † [61]	SCI [c]	Spinal injury level	↑	Treatment with a PTEN inhibitor (TGN)
Myelin-associated proteins				
MAG † [28]	SCI [a]	Lumbosacral spinal cord	↑ *	
MBP † [62]	SCI [a,c]	Undefined (long spinal cord segment)	↓	3-methyladenine
Nogo-A † [28]	SCI [a]	Lumbosacral spinal cord	↑ *	
OMgp † [28]	SCI [a]	Lumbosacral spinal cord	↑ *	
RGMa † [28]	SCI [a]	Lumbosacral spinal cord	↑ *	
Other molecules				
ATF3 ‡ [63]	SCI [c]	L6-S1 DRG	↑	Vibegron
c-Fos • [53,57,58]	SCI [c]	Neuronal voiding centers	↑	Transplantation of oral mucosa stem cells Tamsolusin
		Low lumbar spinal cord (dorsal horn)	↑	Tamsolusin
GAP-43 † [26,27]	SCI [a,c]	Lumbosacral spinal cord [a,c]	↑	
		L5-S1 DRG [a]	↑ *	
GFAP † [37]	SCI [c]	Spinal injury level	↑	
iNOS ‡ [63]	SCI [c]	L6-S1 DRG	↑	Vibegron
LC3-II † [62]	SCI [a,c]	Undefined (long spinal cord segment)	↑	3-methyladenine
NADPH-d • [58]	SCI [c]	Low lumbar spinal cord (dorsal horn)	↑	Tamsolusin
		Neuronal voiding centers	↑	
pJNK † [27]	SCI [c]	Lumbar spinal cord	↑	
S100 † [41]	SCI [c]	Spinal injury level	↑	
Molecular Factors	**NDO Model**	**Location within the Nervous System**	**Expression after NDO Induction**	**Treatments That Reverted or Attenuated NDO-Related Expression Change**
Inflammatory mediators				
IFN-γ † [65]	MS	Brain	↑ (1 w) *	
		Spinal cord	↑ (1 w) *	
IL-1β † [67]	MS	Brain	↓ (10 w)	
IL-4 † [67]	MS	Brain	↓ (10 w)	
IL-5 † [65]	MS	Brain	↑ (1 w) *	
IL-6 † [65,67]	MS	Brain	↑ (1 w) * ↓ (10 w)	
IL-10 † [67]	MS	Brain	↓ (10 w)	
IL-17 † [65,67]	MS	Brain	↑ (1 w) * ↓ (10 w)	
TNF-α † [65,67]	MS	Brain	↑ (1 w) * ↓ (10 w)	
Myelin-associated proteins				
MBP † [65]	MS	Spinal lesion sites	↓	
Other molecules				
GFAP † [65]	MS	Spinal lesion sites	↑	

Table 3. *Cont.*

Molecular Factors	NDO Model	Location within the Nervous System	Expression after NDO Induction	Treatments That Reverted or Attenuated NDO-Related Expression Change
Neurotrophic factors				
NGF • [72]	ICH	Neuronal voiding centers	↑	
Neuronal markers				
TH • [64,73]	PD	Substancia nigra	↓	
		Ventral tegmental area	↓	
Molecular Factors	**NDO Model**	**Location within the Nervous System**	**Expression after NDO Induction**	**Treatments That Reverted or Attenuated NDO-Related Expression Change**
Neuronal markers				
VAChT † [74]	MMC	Dorsal lateral nucleus of the spinal cord	↓	
Other molecules				
GFAP † [74]	MMC	Lumbosacral spinal cord (only E20)	↑	

Many molecular factors have their expression up- or downregulated after NDO induction, depending on the NDO model and on the studied neuronal structure. It is possible to find the most relevant molecular factors explored in the included studies in Table 3, accompanied by treatments that reportedly reverted or attenuated the NDO-related expression change.

Briefly, neurotrophic factors, apoptosis-related factors and ischemia- and fibrosis-associated molecules were upregulated in the neuronal tissues of SCI animals. Inflammatory markers exhibited a tendency to increase shortly after MS induction, followed by a significant decrease from basal several weeks later. Purinergic receptors and transient channels expression showed particularly contradictory results not explained by the NDO model, location within the neuronal system, or molecular analysis technique used. Axonal growth regulators, such as MAG, Nogo-A, and RGMa, were upregulated in the lumbosacral spinal cord of the animals that suffered SCI. Expression of GFAP (a gliosis-associated protein, also used as a marker for astrocytes) was evaluated in SCI, MS, and MMC models and reports indicate it was generally upregulated, particularly near the lesion site.

3. Discussion

Micturition relies on intact communication between supraspinal centers, the spinal cord and peripheral neurons [1]. Connections between the pons, where the pontine micturition center (PMC) is located, and the sacral spinal cord are required for efficient voluntary control over LUT function [4]. Neurologic diseases, including SCI, neurodegenerative disorders (MS or PD), meningomyelocele, and cerebrovascular accidents, may jeopardize urinary function by causing damage to these neuronal circuits [11]. Several studies using animal models of disease have addressed and discussed changes occurring in the bladder and/or the neuronal pathways governing LUT function, contributing to a better understanding of NDO pathophysiology and potential to pinpoint possible future therapeutic targets. The present review systematically analyzed several of these studies and summarized the main findings.

3.1. NDO-Driven Pathology and Induction Model

Any neurological disorder that affects the micturition areas of the central or peripheral nervous system is a possible cause for NDO. We focused on NDO resulting from injury to the CNS as this was the most common situation. Our analysis shows that the vast majority of the animal models used to study NDO are based on SCI models (74%). Models reproducing neurodegenerative disorders, including Parkinson's disease (PD) in 6% of the studies and multiple sclerosis (MS) in 10% of the articles, were less frequently reported. Animal models of meningomyelocele (6%) and stroke (4%) were reported in less than 10% of the studies scrutinized here.

3.1.1. Spinal Cord Injury (SCI)

High-level SCI is followed by a period of little or no bladder reflex activity [22,24], in which the neuronal communication between LUT organs and supraspinal centers is abolished [22]. Spinal shock is gradually replaced by NDO, as a result of the neuroplastic rearrangement of micturition reflexes at the lumbosacral spinal cord [77]. These rearrangements are dependent on C-fibers [11,25,78], which undergo axonal sprouting in the bladder and lumbosacral cord [26,27,79] and lower their threshold [80], resulting in NDO [11,81]. Neuroplastic changes also likely contribute to DSD, which is frequently associated with NDO and leads to increased intravesical pressures and high volumes of residual urine, associated with a high risk of urinary infections and kidney deterioration [82]. SCI was the most-replicated pathology, possibly due to the high reproducibility and homogeneity of experimental procedures and functional outcomes.

The spinal regions most frequently affected in human SCIs are cervical or high thoracic segments, due to abrupt flexion and/or rotation of the head or neck [83]. However, the analysed data indicate that most studies concerning urodynamic problems after SCI relied on low thoracic lesions. As any lesion occurring at the cervical region can result in respiratory compromise and is associated with a high mortality rate due to interruption of the bulbospinal respiratory drive [84,85], lesions of high thoracic or cervical segments are avoided. Instead, most studies refer to injuries between T8 and T10 that also cause NDO without affecting breathing.

Human SCIs mostly occur due to blunt trauma (i.e., motor vehicle crash or sport injuries), where the spinal cord is damaged by an object or displaced bone and/or tissue. Thus, in SCI studies when the goal is to search for post-traumatic lesion-associated processes, repair mechanisms, or test neuroprotective treatments, the preferred method to reproduce SCI is often spinal cord contusion [85]. However, this is not the case when it comes to urological investigations. The majority of the retrieved articles in our systematic study used complete transection models [26–35,38,39,43,44,46,48–50,55,57,62,63] (58%), which may be explained by their ease of reproduction and the lower associated costs, as they do not require specific equipment. One could speculate that, when it comes to studying SCI-induced urinary dysfunction, the chosen method to reproduce SCI is not as important as it is in regenerative or tissue engineering studies. Nevertheless, recent studies have shown that the consequences for urinary function associated with transection and contusion models are, in fact, different [37,86,87]. Although more clinically relevant models, mild contusion models were used only in 20% of the articles in our search [37,45,59–61]. In these cases of mild contusion, most resorted to automatic spinal cord impactors [37,59–61]. These devices present reduced variability between experiments by producing a force-controlled impact, in which the amount of time that the impact tip remains on the tissue is controlled to the millisecond. Additionally, an attached force sensor precisely measures the force of the impact, which minimizes error introduced by specimen movement, and offers the possibility of immediately previewing any problem with the impact [85]. However, these systems are expensive and associated with high maintenance costs. The classical weight-drop method, used uniquely in one study [45], is more affordable and easier to use, though it does not present the same reproducibility, which may translate to a higher number of animals per study [85].

Other SCI protocols include incomplete sectioning of the cord with a scalpel of iridectomy scissors, the most frequent of which are spinal hemisections [42,47,52]. This model is particularly useful in studies in which the goal is to compromise a particular area of the cord. They also simulate more clinically relevant injuries when compared to complete transection, allowing comparison between injured and uninjured fibers in the same individual [88]. However, they do not consider contralateral neuroplasticity and it is more difficult to ensure reproducibility, and additional techniques are necessary to ensure injury consistency between experimental animals [85]. Compression models were the least-reported protocol to induce SCI (7%) [32,36,54,56]. These are helpful to simulate spinal canal occlusion and subsequent ischemia, which are common in clinical injuries.

Based on our data, all of the compression models used an aneurism clip. This technique provides a controlled and highly reproducible injury. It is affordable and presents the possibility of controlling lesion severities by changing the force exerted in the clip and the amount of time the lesion lasts. However, compression models are less controlled than automatized contusion protocols [85].

3.1.2. Multiple Sclerosis

MS was the second most-reported animal model of NDO (10% of the studies) [65–69]. MS is the leading cause of non-traumatic disability affecting the CNS, described as a neurodegenerative auto-immune disorder causing progressive neural demyelination and axonal degradation with a typical relatively early onset [5]. The consequences of MS for LUT function are thought to be attributed to spinal cord demyelination, which likely provokes imbalances between the inhibitory and excitatory neurotransmission between the spinal and supraspinal centers controlling the micturition reflex [89]. MS-related urinary impairments are variable, and likely correlated to the severity of MS phenotype, with detrusor overactivity noted in 50% to 90% of patients, whereas detrusor areflexia is observed in 20% to 30% [90].

Currently, the most commonly used animal model to study MS is the EAE mouse. In these animals, autoimmunity to CNS components is induced through the administration of myelin peptide fragments, which induces a rapid autoimmune reaction directed to the myelin sheath [91]. Our search identified this model as the most prevalent used in urologic investigations [66,68,69], using $PLP_{139–151}$ [66,69] or $MOG_{35–55}$ peptides [68] to initiate an auto-immune response. The use of each of these molecules induces distinct phenotypes with differences in regional/tract specificity, the kinetics of demyelination, and motor neuron involvement [91]. The limitation of this model is related to the discrepancies in the pathogenesis of EAE compared with human MS, as these models are poor in terms of providing information about disease progression and the role of specific T cells in MS pathogenesis. Furthermore, sex- and strain-based differences are observed in the clinical course of EAE, stressing the importance of careful choosing of experimental animals to be used in terms of age and sex.

Another model used to induce MS was the CIE mouse [65,67]. In this case, the MS phenotype was induced by the injection of mouse hepatitis virus (MHV) in a single intracranial injection. The pathology progression is contingent on the amount of the virus introduced, the age of the animal, and the strain of the murine coronavirus used, which permits the control of phenotype progression. The CIE progression phenotype is more similar to the human condition, which constitutes the golden advantage of this model.

3.1.3. Parkinson's Disease

PD models were used in 5% of the analyzed studies [64,71,73]. PD is a neurodegenerative disorder characterized by progressive degeneration of dopamine-producing neurons in the substantia nigra of the midbrain. Together with motor symptoms, PD patients also suffer from lower urinary tract symptoms, present in 38 to 71% of the diagnosed patients [92], most frequently urgency and nocturia [93]. Loss of dopamine in the substantia nigra leads to selective depletion of the same transmitter in the striatum, accompanied by a reduction in the expression of D1 receptors in the same locations. In normal conditions, D1 receptors are involved in the inhibitory mechanisms that control storage periods [94]. Therefore, loss of D1 receptors leads to incontinence. Other PD-like pathologies, such as lesions of basal ganglia, also result in loss of voluntary control over the micturition reflex, leading to uninhibited detrusor contractions at low bladder volumes [92,94].

PD is a multifactorial disease. Most cases are thought to be sporadic, but specific genetic mutations have been linked to familial PD. Animal models for PD investigation can be classified into toxin or genetic models. Toxin-based models induce fast degeneration of the nigrostriatal dopaminergic neurons. In our search, the toxins used to induce PD were 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [73] or 6-hydroxydopamine

(6-OHDA) [71]. Due to their structural similarity to dopamine, these toxins are absorbed by dopaminergic neurons through the dopamine transporter, causing cellular degeneration of these cells [95]. Toxin models are preferred when the goal is to study the consequences of the disease, including urinary dysfunction, rather than its onset, since they are easy to reproduce, and present reduced costs. However, toxin-based models do not fully recapitulate human PD, which has a slow and progressive onset. In these regards, genetic models are more suitable, as they provide a more realistic, human-like disease onset, offering tools to study the molecular mechanisms associated with pathology onset. Nevertheless, our search found just one study relying on genetic models, in which the GM2 gene was deleted in mice [64].

3.1.4. Meningomyelocele

Meningomyelocele was investigated in 6% of our retrieved articles [74–76]. This pathology is the most severe type of spina bifida, a congenital neurological abnormality occurring when the spinal cord does not form properly due to defective closure of the caudal neuropore of the neuronal tube. As the spinal nerves controlling bladder function do not form correctly, meningomyelocele is accompanied by neurologic bladder symptoms [96], including NDO and DSD. To induce meningomyelocele, studies resorted to pregnant female rats, intragastrically injected with retinoic acid on embryonic day 10. This model is capable of reproducing the entire spectrum of severity observed in human meningomyelocele, ranging from exposure of the cord with intact neural elements to complete cord destruction [97].

3.1.5. Cerebral Vascular Accidents

Cerebral vascular accidents were the least-used animal NDO models in our search [70,72]. However, more than half of stroke patients, either ischemic or hemorrhagic, report symptoms of urinary dysfunction, including urinary urgency, frequency, and urge incontinence. The presence of DSD is also encountered [98]. These urodynamic symptoms may be present within 72 h of the cerebrovascular accident and, in 30% of the patients, within four weeks after that time point [99]. In our search, we found two methods to induce stroke: middle cerebral artery occlusion (MCAO) and enzymatic induction of cerebral hemorrhage. The MCAO model, used to study ischemic stroke [70], is achieved by the insertion of a filament in the middle cerebral artery, which is removed afterwards. This produces a transient ischemia followed by the restoration of blood circulation, as it happens in humans. This method avoids the need for craniotomy and its possible negative effects on blood–brain barrier permeability and intracranial pressure [100]. However, MCAO may cause subarachnoid hemorrhage, tracheal edema, and paralysis of the muscles of mastication and swallowing if damages in the external carotid artery occur [100]. The other method referred to is the induction of cerebral hemorrhage. In this case, the hemorrhage is induced by a collagenase injection in the hippocampal CA1 region [72]. Collagenase enzymatically disrupts the basal lamina of blood vessels, causing an active bleed into the surrounding tissues that generally evolves over several hours. Both methods can be adapted to injuries in any brain region.

3.2. Animal Species

Our search demonstrated that rodents were used in 95% of the retrieved articles concerning NDO. Rats were the most commonly used (73%), followed by mice (22%). Rats have the advantage of low maintenance costs, ease of care, and a well-studied anatomy [101]. Their bigger size, when compared to smaller rodents, allows for more complex surgical interventions, which is particularly important in models based on the physical lesioning of CNS areas. Several established behavioral tests, which are used to assess the loss and recovery of neurologic deficits, are better adjusted for rats than other rodents. Concerning urodynamic testing, the majority of established techniques are better studied and established in rats, providing superior testing outcomes and numerous sources of comparison [102].

However, mice models are becoming more popular and increasingly implemented in NDO studies. Morphologically, the mouse bladder appears to be more similar to humans, but the urodynamic properties of the mouse LUT have not been characterized as well as those of rats [103]. Mice offer the possibility of generating genetically modified models and have higher reproductive rates and low maintenance costs. The disadvantages of using mice are related to their smaller size, which poses problems for several induction protocols and urodynamic recordings and urethral electromyography [103].

Despite the benefits of using experimental animals to investigate NDO pathophysiology and test new therapeutic approaches, the results should be approached with caution. It is important to note some morphological and physiological differences. In rodents, the prostate is not encapsulated within a well-formed prostatic fascia [104]. Additionally, the architecture of the pelvis and pelvic floor corresponds directly to the quadruped locomotion of rodents, which is different between the two species. Functionally, there is evidence that detrusor contraction in rodents is dependent on ATP acting as a neurotransmitter, whereas in humans, it is mediated by acetylcholine [103]. These differences may affect the functional outcomes [104].

Other animal models, such as rabbits and non-human primates, may provide a more physiologically relevant evaluation of outcomes compared to rodents, particularly considering the similar size of the spinal cord, comparable neurological damage mechanisms, and higher anatomical parallel. However, the use of these non-rodent animal models is limited by maintenance costs and strict ethical requirements. Accordingly, our systematic search encountered three studies using rabbits [33,41], and one using the marmoset [73]. No studies with primates were reported.

3.3. Animal Sex

Our analysis demonstrated that more than 70% of the studies favored females to induce NDO. This is likely related to the feasibility of transurethral catheterization and manual bladder emptying, since the male urethra is surrounded by the prostatic gland, which makes abdominal compression and bladder manipulation more difficult in males [33,104]. Nevertheless, sexual dysmorphism in micturition behavior should be accounted for. In addition, one should not forget that some human pathologies might be more prevalent in one **sex** than the other, making data obtained in studies using only female or male animals more difficult to clinically translate. Experiments concerning the effect of the estrous cycle on rat bladder contractility have pointed to a more responsive behavior of females bladders [105] compared to males in response to cholinergic stimulation. This likely reflects **sex**-related differences in bladder expression of different subtypes of muscarinic and adrenergic receptors [106–108]. Accordingly, the cholinergic neurotransmission is predominant in the male bladder, while the purinergic component is prevalent in females [109]. Sex differences were also noted in expression of acid-sensitive ion channels (ASICs) and transient receptor potential vanilloid type 1 (TRPV1), both key channels for normal and pathologic bladder function [110]. These molecular discrepancies are likely reflected in bladder function.

Micturition patterns are also different between male and female experimental animals. Male voiding consists of a fast spike-like urine flow, whereas female voiding is ongoing but interrupted for short periods when bladder pressure is increased. The maximum flow rate is lower and voiding period is shorter in female rats as compared to male rats [111]. These dimorphic micturition patterns in rats might be attributed to the different nature of the perineal muscles of the EUS, less prominent in females [112]. Though these differences are considered to be of minor relevance in normal function, they might have a significant impact in pathologic conditions. To understand the real impact of sex on pathologic LUT function, research studies would benefit from using both male and female animals. However, this was only the case in two studies [64,73], both concerning PD models.

3.4. Urodynamic Recording

Changes in LUT function are evident after induction of SCI, MS, PD, meningomyelocele, or stroke. Animals present typical NDO symptoms, including increased voiding frequency, basal pressure, maximum voiding pressure, threshold pressure, and high amounts of residual urine. As result, the voided volume per contraction is reduced and a significant decrease in voiding efficiency is evident. Some studies also reported the presence of DSD. The majority of the retrieved articles evaluated the consequences on LUT function by using urodynamic recording techniques.

For decades, the gold standard method to perform urodynamic evaluation in animals has been using cystometry under urethane anesthesia. This drug is the most-used anesthetic for urodynamic recording, as it is recognized as the most preservative of micturition reflexes. Nevertheless, urethane interferes with urethral sphincter activity, resulting in reduction in voiding efficiency and increase in post-void residual volume [113–117]. Moreover, urethane anesthesia is limited to terminal procedures, due to its adverse post-operative health effects and carcinogenic risks [118]. One could speculate that recent papers would resort to better anesthetic options, but urethane remains the main choice for urodynamic evaluations [26–28,37,41,43,51,57], as it has low associated costs, it is easy to deliver, and there are abundant published references using this anesthetic for cystometries [102,119].

Fewer studies have used zolotyl anesthesia as an alternative for urethane [53,58,61,74]. Zolotyl is a combination of tiletamine and zolazepam and was used in 8% of the articles in our search. Zolotyl produces a smooth conscious sedation, characterized by a rapid induction period, together with excellent muscle relaxation with a wide safety margin, and smooth recovery [120]. One article included in our search used chloral hydrate as an alternative to urethane to perform cystometries [54]. This anesthetic is no longer recommendable to use, due its toxic components.

In an attempt to overcome the negative effects of anesthetics on LUT function, awake cystometries have arisen as a popular method to record bladder and urethral function in rats and mice. Unlike anesthesia protocols, in which environmental cues and diurnal variations are suppressed, it is necessary to consider that cystometries in awake conditions are influenced by external factors, such as light and noises. Furthermore, as rodents are nocturnal, the experiments must be performed during night time, or animals must be acclimatized to inverted cycles of light [102]. The variety of awake recording systems range from restrained to freely moving animal approaches [121]. In restrained animals, it is easier to manage the position of the intravesical catheter, and to prevent the occurrence of urodynamic artifacts. However, despite pre-testing habituation to the cystometry stations, restraining may cause high levels of stress, which can increase sympathetic activity, favoring storage and potentially prolonging the time of bladder filling until micturition [122]. Unrestrained conditions, using metabolic cages, closely resemble physiological conditions and are assumed to better record LUT function [123], but studies using this approach are still scarce.

For urodynamic assessment in experimental animals, it is necessary to place an intravesical catheter that allows for saline injection and/or recording of bladder contractions. These catheters can be placed acutely or be indwelling. Acutely placed catheters are suitable for terminal procedures when the recordings occur right after implantation surgery and the animal is euthanized immediately after recording. There are, however, disadvantages linked to the use of acute catheters, such as postoperative pain and the fact that the anesthetics used in the surgical procedure might affect LUT activity [102,122]. An alternative for this is the use of chronic indwelling catheters and electrodes for urethral electromyography, which can be externalized on the animal's dorsum and maintained for large periods, permitting animal stabilization after surgery. Additionally, they also allow the testing of the same animal several times during the experimental protocol, eliminating inter-animal variability and reducing the number of animals required [124]. However, these systems are associated with a high maintenance cost and complex post-operative care to maintain the

functionality of the externalized components for extended periods and the wellbeing of the animals [102,124].

While urodynamic recording is the only method that can objectively assess lower urinary tract function, non-invasive methods, including the voiding spot assay, were also reported in our search [64,65,67,68]. The voiding spot test has the advantage of being minimally invasive, inexpensive, and easy to implement. However, the urodynamic data are poor, only recording voided volumes and spatial and temporal organization of urinary spots [102]. Surprisingly, this was the preferred method to evaluate bladder changes after induction of neurodegenerative disorders [64,65,67,68].

Surprisingly, a significant portion of retrieved studies did not present any urodynamic data [29,30,36,44,45,49,50,60,62,66,69,73–76]. This was quite unexpected for studies with a focus on urinary dysfunction and NDO. One could speculate that these studies used animal models that are already established, so their effects were already known and described. These studies focused on other aspects of the disease than urinary function, including molecular alterations. In fact, the lack of any evaluation of LUT function was seen in studies using animal models of myelomeningocele, in which urodynamic recording would be difficult to perform.

3.5. Changes in Bladder and Neural Tissue Morphology

Selected studies highlighted significant findings regarding bladder and neuronal tissue morphology. Bladder tissue was generally more fibrotic in NDO animals, in tandem with findings in humans [125]. Histologically, bladder fibrosis is described as an increase in connective tissue elements, particularly in the detrusor, where collagen fibers heavily surround smooth muscle cells. These changes are driven by several molecular factors, which are upregulated in NDO bladders, and represent tissue remodeling following DSD and consequent bladder volume load increase. This functional obstruction also leads to detrusor smooth muscle hypertrophy, chronic inflammation, and edema. All these features result in an increase in bladder weight, reflecting bladder wall thickening [59]. Smooth muscle ultrastructural changes after SCI-inducted NDO were also found in the retrieved studies, such as mitochondrial swelling and endoplasmic reticulum hypertrophy [54], consistent with smooth muscle hypertrophy and increased intensity of bladder contractions. The mucosa, particularly the urothelium, also undergo plastic changes, shown to contribute to impaired urinary function in NDO models [126].

Concerning neuronal tissue, various CNS and PNS structures are affected, depending on the NDO model. SCI was the model that presented greater morphological changes, with formation of fibrotic scars at the injury site, associated with recruitment microglia, astrocytes, macrophages, and other inflammatory cells. These cells eventually fill the injury core and are involved in complex crosstalks to repair the injured tissue but prevent axonal regrowth [127]. Because SCI is the most-used method to induce NDO, the considerations below mostly refer to SCI.

3.6. Molecular Factors

Molecular changes in the bladder and neuronal tissue after NDO induction are, respectively, presented in Tables 2 and 3. These variations were detected through either protein or RNA analysis. The majority of data gathered was obtained from SCI studies.

3.6.1. Neurotrophic Factors

Neurotrophic factors are growth factors that play a critical role in neuron survival and regeneration, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial cell-derived neurotrophic factor (GDNF) [128,129]. NGF is a small molecular weight protein, involved in urinary dysfunction in several contexts, including SCI [130,131]. In the bladder, NGF is secreted by smooth muscle and urothelial cells [132–134], and its levels are increased in response to inflammation or denervation [135]. In animal models of NDO, bladder NGF levels also vary, increasing after SCI [29,46,48,56]

and being reduced in CI and MS animals [66,70]. In the latter, the time point studied referred to chronic stages of disease progression and it is not possible to exclude increased NGF levels in the acute phase of the MS model [66]. Importantly, while it is possible to observe that, as in cystitis [134,136,137], SCI-induced NDO courses with high NGF levels [138–140], the same does not happen in MS models. While this likely reflects different pathophysiological mechanisms for NDO, the precise reasons can only be speculated at present. Importantly, high levels of bladder NGF coursed with upregulation of the phosphorylated form of the high-affinity NGF receptor TrkA, which was also observed in PD models [29,64].

In neuronal structures, NGF was also upregulated after NDO induction, when quantified in nervous system structures, such dorsal root ganglia, supraspinal neuronal voiding centers and the spinal cord [48,53,57,61,72]. Nevertheless, it is important to point out that this analysis was not performed on an SCI or on an MS model. Regarding BDNF, protein expression was increased in SCI animals, both in the bladder and the spinal cord [27,61], but variations of its levels in other models were not found. Changes in GDNF levels were only reported in an MS model, in which the bladder contents were found to be reduced [66]. Such changes in neurotrophic factors are likely involved in the abnormal axonal sprouting resulting in expansion of C-fibers in the bladder wall and lumbosacral spinal cord, a key event in NDO development and maintenance [12,26,27,80].

3.6.2. Inflammatory Mediators

Changes in inflammatory molecules, including pro- and anti-inflammatory cytokines, were described in articles using MS animal models. These mediators, such as IL-2 and TGF-β, were dramatically increased in the bladder tissue [59]. In neuronal tissue, the variation in expression levels of cytokines is complex to analyze. Inflammatory cytokines were increased after 1 week but decreased 10 weeks after MS induction [65,67]. The first week period in animal MS models may represent an acute immune event, linked to inflammatory demyelination. The reduction at 10 weeks likely reflects modulation of immune responses. These changes coursed in NDO installation and likely reflect changes in immunological activation associated with MS.

3.6.3. Apoptosis-Related Factors

Apoptosis is the process of programmed cell death, involving several players in complex pathways, including enzymes such as caspases. In animal models of SCI, Caspase-3 was found to be activated at the injury site, since trauma and ensuing events lead to cell death of resident and invading cells [127]. After traumatic SCI, spinal tissue at the injury site undergoes major transformations. Healing is a complex process that results in tissue remodeling, which seals the injured location. Traumatic SCI causes direct tissue destruction (compression, laceration, shearing of the cord) that results in profound histological modifications at the injured location [141–143]. This is followed by production of free radicals, lipid peroxidation, altered ATP production, invasion of peripheral immune cells (including neutrophils, lymphocytes, and monocytes) due to breakdown of the blood–brain barrier, activation of resident astro- and microglia, and neuronal and glial apoptosis, all of which contribute to further damage of the injured area [142]. The final step consists of the formation of a glial scar, highly repulsive to axonal growth, preventing appropriate rewiring, reestablishment of connections between supraspinal centers and lumbosacral neurons and, ultimately, full recovery [142]. While apoptosis is central at the injury site within the spinal cord, we found no reference to a direct link with NDO development or maintenance. No studies addressed the presence of pro-apoptotic elements in the bladder of NDO animals.

3.6.4. Muscarinic Receptors

Muscarinic receptors play an important role in detrusor contraction and they can be found in the detrusor layer and the mucosa, participating in the urothelium–detrusor

crosstalk and regulating detrusor contraction [144,145]. The mRNA levels of M2 and M3 muscarinic receptors in the bladder mucosa of SCI animals (6 weeks after lesion) were downregulated, but only M2 protein levels were reduced when compared to controls [44]. Another study with rodents, not included in this review [144], showed an increase in M2 subtype transcript in the bladder mucosa 2 weeks after SCI, returning to basal by 4 weeks. There was no similar pattern in the M3 subtype. Detrusor protein expression of M2 receptors increased during chronic SCI period and the M3 subtype was downregulated [56]. In animals with cerebral ischemia, M3 was downregulated [70], but there are conflicting observations regarding M2 levels, possibly reflecting different analytic techniques [70]. Changes in the expression of muscarinic receptors may underly the lack of response of patients to anti-muscarinic therapy. This is relevant as treatment of NDO is typically initiated with anti-muscarinic drugs [9,16]. If patients do not respond to low amounts of these drugs, the dosage is increased, but only refractory patients will receive botulinum toxin A as the last-resort treatment [146,147].

3.6.5. Adrenergic Receptors

In terms of adrenergic receptors, the retrieved studies documented changes in their expression in the bladder of SCI animals presenting NDO. In the clinical setting, α1a adrenergic receptor (AR) antagonists have been used in multiple pathologies, such as prostatic benign hyperplasia [148] and DSD after SCI, to produce muscle relaxation and decrease urethral sphincter pressure and obstructive symptoms [60]. However, this therapy is not always fully effective, which likely reflects the downregulation of α1a adrenergic receptor expression after SCI [60]. The expression of β2-adrenergic receptors in the bladder was also studied in the context of SCI and a reduction in the bladder levels of β2-adrenergic receptors was found [56].

3.6.6. Purinergic Receptors and Transient Receptor Potential Channels

The importance of P2X and TRP receptors in urinary function is well established [149]. We found two contradictory perspectives on the expression of P2X purinergic receptors and TRP channel expression in SCI animals. Concerning the expression of these receptors in DRG cells, four studies [35,46,49,63] reported upregulation of TRP and P2X receptors, while another study [45] reported TRP/P2X pathway elements' downregulation. Similarly, in the bladder, three studies [30,51,56] showed upregulation of TRP and P2X elements, while one reported downregulation [45]. These different observations likely originate from different methodologic approaches, as the majority of available studies refer to the upregulation of these ion channels as key events to explain enhanced excitability of C-afferents, known to be a driver of NDO development and maintenance in SCI [11,12,78].

3.6.7. Neuronal Markers

Analysis of β-III-tubulin, a pan-neuronal marker, in 9-week SCI rats shows upregulation of this protein in bladder tissue, indicating the occurrence of hyperinnervation in the bladder wall and demonstrating neural plasticity and compensatory axonal regeneration [36]. This agrees with increased bladder levels of neurotrophins, such as NGF, which induce axonal growth and branching [130,131].

In the nervous system of NDO rats, the expression of several neuronal markers (namely CRF, GAD2, NF200, TH, VAChT, and VGLUT) was found to be generally downregulated, due to denervation associated with SCI, MMC, and PD, the latter more evident in the substancia nigra and ventral tegmental area [40,47,49,64,73,74]. In contrast, the expression of CGRP, a marker of sensory innervation, was upregulated in the lumbosacral spinal cord and L1 and L6 DRGs in SCI animals, in tandem with what has been described in the bladder [26] and coursing with levels of spinal NGF [140].

3.6.8. Ischemia- and Fibrosis-Related Molecules

This category includes not only molecular factors that play a critical role in the setting of fibrosis, such as CTGF, FGF, and TGF, but also a broader group of molecules responsible for ischemic response, such as HIF and VEGF. All were upregulated in the bladder and neuronal tissue in SCI and MS models. This indicates that, in both conditions, NDO development and maintenance are associated with intensive tissue remodeling [59].

Astrocyte-derived chondroitin sulphate proteoglycans (CSPGs)—phosphacan and neurocan—were also included in this group, since they are the central extracellular matrix components of the spinal fibrotic scar that seals the injury site after SCI [28,127]. The levels of CSPGs are highly increased at the injury site after SCI [142,150], correlating with upregulation at the same location of S100, a glial marker [41], consistent with the accumulation of glial cells and scar formation [142,150]. Importantly, CSPGs are also elevated in in segments distant from the scar [28,151], indicating a widespread response to SCI. While this upregulation of CSPG content is exhuberant at the injury site, it is more controlled and restricted in segments distant from the injured tissue, as only the expression of specific CSPGs is changed in a time-dependent manner [28,151]. CSPGs are known to be involved in axon guidance regulation [152] and it is possible that this lumbosacral upregulation might be linked to the establishment of new neuronal circuits responsible for abnormal bladder function after SCI.

3.6.9. Myelin-Associated Proteins

This group of molecules can be divided in two different clusters: proteins associated with the myelin sheath, such as myelin basic protein (MBP), and myelin-associated inhibitory proteins (MAIs)—MAG, Nogo-A, and OMgp. MBP was downregulated in the spinal cord, both after SCI and MS. MBP downregulation possibly reflects loss of myelin sheaths due to apoptosis of neurons and oligodendrocytes secondary to SCI [62], while its downregulation in MS models can be explained by CNS demyelination. Concerning MAI expression in the lumbosacral cord, levels of Nogo-A were transiently upregulated, without changes in MAG and OMgp, after thoracic SCI [28]. Changes in the expression of these guidance molecules may well be linked to neuroplastic events leading to NDO establishment.

4. Conclusions

NDO is a common consequence of neurologic injuries, with a tremendous impact on the quality of life of affected patients. Animal models of NDO have been critical for understanding the pathophysiology of the disease, as well as to study potential for recovery and implement new therapeutic targets for affected patients. In this review, we describe the currently used animal models to study NDO, and discuss them in terms of species, sex, urodynamic recordings, and molecular alterations observed in bladder and neuronal tissue. Despite the heterogeneity of NDO onset, the vast majority of studies concerning molecular mechanisms associated with this pathology are based on traumatic SCI models. However, NDO is also a consequence of several progressive diseases such as neurodegenerative disorders, meningomyelocele, and stroke, about which there is much less information. It is important that future studies focus on these disorders to provide a better understanding of the pathophysiological mechanisms leading to NDO, which is important for the development of new therapies targeting these patients' quality of life. The list of molecular changes found in the present review is vast and includes the upregulation of inflammatory mediators, molecular markers of fibrosis. Moreover, there is also significant evidence of neuronal plasticity with increased expression of neuronal receptors, neurotrophins, and myelin-associated proteins both in the bladder and neuronal tissue, supporting the wide range of neuroplastic events that result in NDO. While it is difficult to grasp and integrate the enormous number of published results, it is clear that NDO pathophysiology is complex and, consequently, its treatment and management is difficult. Like other researchers [18] and following the present review, we propose that

many key players are active and interacting at different stages of disease progression. It is likely that future interventions will result from the combination of different drugs simultaneously targeting different molecules. Future research should use comprehensive strategies, possibly automated, to identify synergistic changes and key events that could be therapeutically targeted.

5. Materials and Methods

5.1. Literature Search

The present systematic review was elaborated following the PRISMA 2020 checklist [153]. We aimed to analyze scientific articles that addressed molecular changes associated with neurogenic detrusor overactivity. On 19 September 2022, the search was conducted in three databases: PubMed Central (via PubMed), and Medline and Embase (via Scopus). The following query was used: ((Animal model) OR (rat) OR (mice) OR (rabbit) OR (pig)) AND ((Neurogenic detrusor overactivity) OR (neurogenic bladder)). No filters were used, and the search was limited to articles published between 1 January 2012 and 19 September 2022. The year 2012 was chosen as a reference as it was the year in which some automated devices for spinal contusion became commercially available [154]. This search generated 648 results.

5.2. Selection

The studies retrieved were imported to Endnote and duplicated articles were excluded. The remaining articles were then imported to the Rayyan platform, and the remaining duplicates (not detected by Endnote) were identified and excluded. The resultant articles (365) were submitted to title and abstract screening, independently conducted by two investigators. The inclusion criteria were: (1) studies including an animal model of neurogenic detrusor overactivity; (2) studies including at least one molecular analysis technique; and (3) studies published in English. The exclusion criteria were: (1) non-original studies, such as reviews, conference abstracts, and editorials; (2) studies conducted in humans, such as case reports and clinical trials; (3) in vitro studies; (4) absence of data on molecular alterations; (5) studies using an SCI model which merely present molecular results obtained from animals euthanized 14 days after SCI induction (acute SCI); (6) studies focusing on NDO of peripheral origin; and (7) impossibility of obtaining the full-text article (even after contacting the authors). All articles were submitted to full-text screening if no inclusion criteria were absent and if no exclusion criteria were met.

To assess the risk of bias in our work, SYRCLE'S risk of bias tool was used. This checklist was adapted from the Cochrane risk of bias tool and adjusted for experimental animal studies. The tool was developed by Hooijmans et al., and focuses on evaluating selection bias, performance bias, detection bias, attrition bias, and reporting bias in animal experimental studies [155]. The Cochrane risk of bias (Rob) checklist was also consulted [156].

5.3. Data Extraction

Outcomes for which data were sought were the following: (1) study characteristics (author and year of publication); (2) used model of neurogenic detrusor overactivity; (3) used animal species; (4) animal sex; (5) model induction method; (6) urodynamic findings; (7) changes in bladder tissue; (8) changes in neuronal tissue; and (9) therapies and mechanisms identified. If any of these study characteristics were not evident from full-text analysis, authors were contacted. Basic study characteristics, such as the animal sex, are described as unknown if there was no answer from the authors. All gathered data have been included in Table 1. Two independently working reviewers extracted the most relevant data from every included article. No automatic tools were used.

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Urinary dysfunction after spinal cord injury: Comparing outcomes after thoracic spinal transection and contusion in the rat

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ABSTRACT

Spinal cord injury (SCI) above the lumbosacral spinal cord induces loss of voluntary control over micturition. Spinal cord transection (SCT) was the gold standard method to reproduce SCI in rodents, but its translational value is arguable and other experimental SCI methods need to be better investigated, including spinal cord contusion (SCC). At present, it is not fully investigated if urinary impairments arising after transection and contusion are comparable. To explore this, we studied bladder-reflex activity and lower urinary tract (LUT) and spinal cord innervation after SCT and different severities of SCC. Severe-contusion animals presented a longer spinal shock period and the tendency for higher residual volumes, followed by SCT and mild-contusion animals. Urodynamics showed that SCT animals presented higher basal and peak bladder pressures. Immunostaining against growth-associated protein-43 (GAP43) and calcitonin gene-related peptide (CGRP) at the lumbosacral spinal cord demonstrated that afferent sprouting is dependent on the injury model, reflecting the severity of the lesion, with a higher expression in SCT animals. In LUT organs, the expression of GAP43, CGRP cholinergic (vesicular acetylcholine transporter (VAChT)) and noradrenergic (tyrosine hydroxylase (TH)) markers was reduced after SCI in the LUT and lumbosacral cord, but only the lumbosacral expression of VAChT was dependent on the injury model. Overall, our findings demonstrate that changes in LUT innervation and function after contusion and transection are similar but result from distinct neuroplastic processes at the lumbosacral spinal cord. This may impact the development of new therapeutic options for urinary impairment arising after spinal cord insult.

Introduction

Normal micturition relies on synchronised contraction and relaxation of the two functional units of the lower urinary tract (LUT): the urinary bladder and the urethra. Such coordination relies on intricate neuronal circuits involving afferent and efferent pathways as well as supraspinal centres. Spinal cord injuries (SCI) rostral to the lumbosacral spinal cord eliminate voluntary supraspinal control of micturition. SCI is followed by a period of little or no bladder reflex activity, termed spinal shock, with partial or full urinary retention during the immediate days/weeks after spinal insult (Anderson et al., 2023; Bywater et al., 2018). Later, as neuroplastic mechanisms operating at the lumbosacral spinal cord are established, including sprouting of afferent fibres and establishment of new synaptic contacts, an alternative micturition reflex pathway, independent from supraspinal input, emerges (de Groat and Yoshimura, 2012; Wada et al., 2022). Micturition becomes involuntary and largely inefficient due to strong and frequent detrusor contractions, demonstrated by urodynamic evaluation, giving rise to neurogenic

Abbreviations: CGRP, calcitonin gene-related peptide; DSD, detrusor sphincter dyssynergia; GAP43, growth-associated protein-43; LUT, lower urinary tract; NDO, neurogenic detrusor overactivity; SCI, spinal cord injury; TH, tyrosine hydroxylase; VAChT, vesicular acetylcholine transporter; SCT, spinal cord transection; SCC, spinal cord contusion; mSCC, mild contusion; sSCC, severe contusion.

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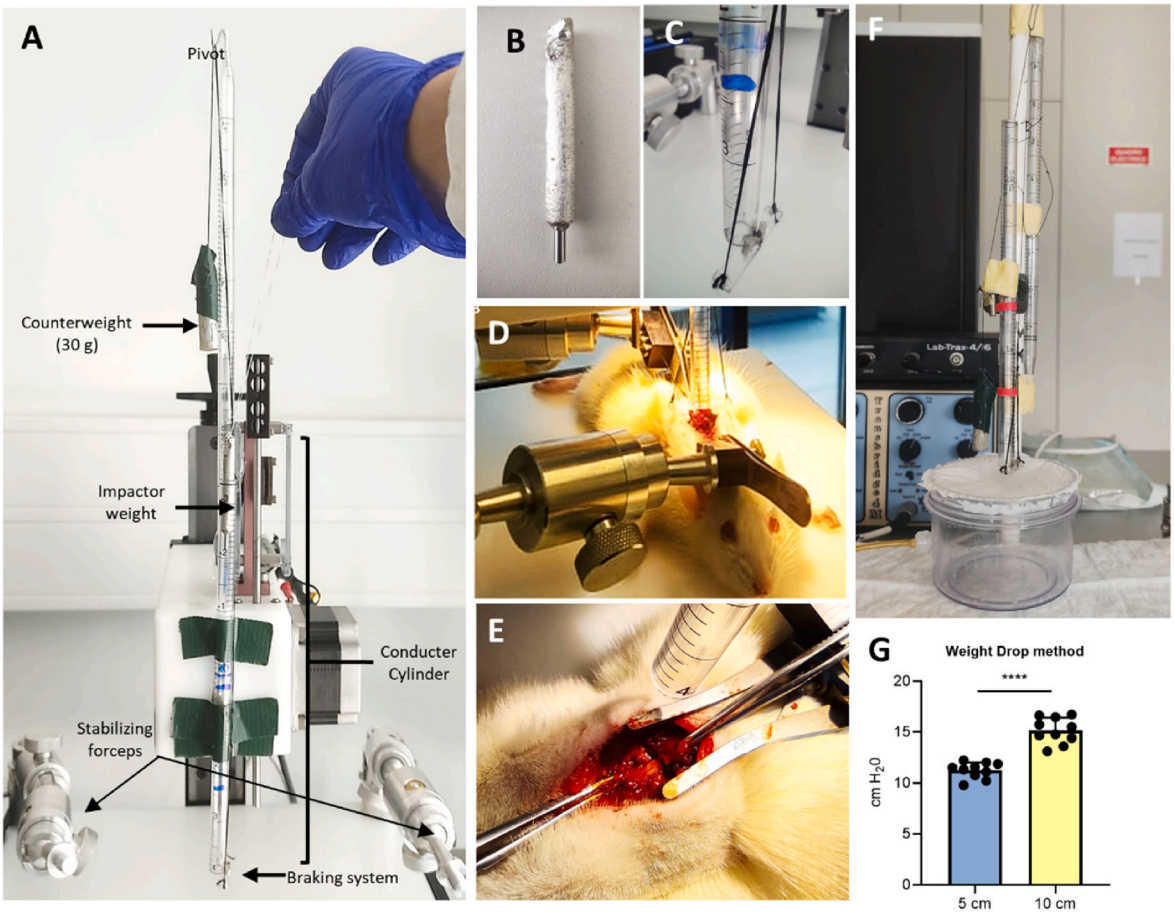

Fig. 1. Contusion apparatus. (**A**) The contusion system incorporates a rigid surface with attached forceps to stabilize the vertebrae of the animal during impact. The device is attached to a manipulator that allows adjustments of the position of the impactor tip in relation to the animal's exposed spinal cord. The weight drop device consists of a 20 cm cylindrical tube with 6 mm in diameter attached to the manipulator's arm. (**B**) The weight is a small steel bar (10 g, 4.5 cm long) with a 2.5 mm diameter tip. (**C**) The braking system is composed of a brake (a 2.8 cm long piece of plastic, adapted with a 0.5 cm lock in the centre placed at the end of the conducting cylinder, which is linked to the impactor weight and a counterweight (30 g). (**D**) The caudal and rostral vertebrae of the animal should be clamped, to ensure the animal is immobilised during impact. (**E**) For an appropriate impact, it is important to expose the spinal cord. (**F**) To validate the device, a plastic jar with acovering silicone membrane. A pressure transducer was inserted in the silicone cover and connected to a pressure transducer and an amplifier, to record the pressure of the membrane before and after weight drops. (**G**) The average impact force produced was greater when the metal rod was dropped from 10 cm high, compared to when the weight was dropped from 10 cm high. The standard deviation between trials was small, validating the reproducibility of the model. Data were compared with a *t*-test (**** $p < 0.0001$).

detrusor overactivity (NDO) (Gajewski et al., 2018). NDO is often concurrent with detrusor sphincter dyssynergia (DSD), defined as a loss of coordination between the detrusor and the urethral sphincter (Stoffel, 2016). Consequently, the bladder may not be efficiently emptied, causing bladder enlargement, due to an accumulation of high volumes of residual urine, giving rise to dangerously high intravesical pressures (Wada et al., 2022; Wyndaele, 2016) which may be deleterious to the upper urinary tract.

The reorganisation of the lumbosacral neuronal pathways governing micturion following SCI is accompanied by noteworthy alterations in innervation and histology of LUT organs (Ferreira et al., 2022; Shimizu et al., 2023), including neuronal sprouting at the spinal cord (Frias et al., 2015; Oliveira et al., 2019; Vizzard, 1999), increase in neurotrophic factors secretion (Keefe et al., 2017; Ochodnicky et al., 2012; Vizzard, 2006), and changes in the proprieties of LUT afferents (de Groat and Yoshimura, 2006). Morphological changes in the bladder have also been described both in the mucosa (Apodaca et al., 2003; Birder, 2006; Kullmann et al., 2017) and detrusor muscle (Johnston et al., 2012; Oliveira et al., 2019). Alterations in the urethra in response to SCI are less documented, but we have recently demonstrated marked urethral epithelium reorganization accompanied by sphincter atrophy and a pronounced decrease in the expression of general innervation markers, particularly markers of sensory and noradrenergic nerve fibres (Ferreira et al., 2022).

Much of our knowledge about SCI-induced generated urinary dysfunction has been obtained in studies using spinal cord sectioning. Indeed, complete spinal cord transection (SCT) and spinal hemisection have been the most commonly used experimental SCI protocols (Ferreira et al., 2023; Sharif-Alhoseini et al., 2017; Verstappen et al., 2022), with complete SCT being traditionally prefered for many years in studies focusing exclusively on bladder function (Cheng et al., 1999; Cruz et al., 2006a; de Groat et al., 1990). In addition to allowing the identification of supraspinal contribution to the regulation of bladder function, complete SCT also has the advantage of being inexpensive and easily reproducible. However, complete spinal lesions are rarely seen in clinical practice and the translational value of SCT as a model of disease is debatable. Indeed, spinal contusions are the most frequent causes of human SCI and lesions are often incomplete (Liu et al., 2019; Theodore, 2016). Experimental contusions are currently seen as more relevant in SCI research (Blight, 2000; Sharif-Alhoseini et al., 2017) despite carrying some inter-trial variability (Cheriyan et al., 2014; Sharif-Alhoseini et al., 2017). While complete SCT has been classically used to study SCI-induced bladder dysfunction, as contusions become a more commonly used model, it is important to compare both experimental

approaches in what concerns bladder function and changes in neuronal content at both the LUT and lumbosacral spinal cord levels. This would confirm if data obtained in SCT studies can be extended to models of spinal contusion, a matter we investigated in the present study.

Methods

Animals and drugs

In-house-bred female Wistar Han rats (200–250 g; n = 4–5 per experimental group; derived from Charles River Laboratories, France). To prioritize animal welfare and considering anatomical differences, including shorter urethra and absence of prostate, female animals were preferred due to the relative ease of manually emptying their bladders during spinal shock, when there is little or no bladder reflex activity able to promote voiding. While we recognize the use of male animals should be considered in terms of representation of human injuries, male SCI rodents are more prone to urinary retention, present higher risk of bladder infection, urethral blockages, and lethal kidney failure. Animals were maintained under a 12 h light/dark inverted schedule and controlled temperature and air humidity, with *ad libitum* access to food and water. Experimental procedures were carried out per the European Communities Council Directive 2010/63/EU, to ethical guidelines for investigating pain in animals and internal regulations of the Faculty of Medicine of Porto. The ARRIVE guidelines have been followed during the course of this study. Animals were randomly divided into four groups: SHAM (controls), complete spinal cord transection (SCT), mild contusion (mSCC) and severe contusion (sSCC). All efforts were made to reduce the number of animals used and their suffering.

Drugs

All surgeries were performed under deep anaesthesia, induced with an intraperitoneal (IP) injection of medetomidine (0.5 mg/Kg) and ketamine (75 mg/Kg). Anaesthesia was reverted with an intramuscular injection of atipamezole (1 mg/Kg). After surgery, all animals received 5 % saline-glucose (1 mL, IP) to compensate for blood loss and dehydration. Animals also received oral antibiotics (enrofloxacin; 5 mg/Kg). Antibiotic administration was initiated on the day of surgery and maintained for 8–10 days. For pain management, rats were treated with subcutaneous buprenorphine (0.02 mg/Kg) for 3–4 days after surgery and whenever deemed necessary, after that time point. For euthanasia, animals received an intraperitoneal injection of sodium pentobarbital (56 mg/g).

Spinal cord contusion device

The device used in the present study was constructed based on previously used setups (Lima et al., 2020; Vasconcelos et al., 2016). To ensure stability, the device was placed on a rigid and stable surface (Fig. 1A). As spinal trauma typically causes involuntary reflexes, this rigid surface has attached forceps to allow steadying of the vertebrae during impact (Fig. 1A). The device itself consists of a 20 cm cylindrical hollow tube (adapted from a 10 mL plastic pipette) attached to a manipulator's arm, which allows the adjustment of the set-up to the animal's position (Fig. 1B, 1D). The tube conducted the weight onto the exposed spinal cord. The weight consists of a 4.5 cm long metal cylinder, weighing 10 g, with a 2.5 mm diameter tip (Fig. 1B). The device used has an incorporated braking system, important to control the amount of time the weight's tip rests on the exposed spinal cord, avoiding dissimilar functional outcomes. The brake allows for a more controlled weight drop and consists of a 2.8 cm long piece of plastic, with a 0.5 cm lock in the centre, placed at the end of the conductive cylinder (Fig. 1C). A 30 g counterweight is also necessary to revert the weight's trajectory once it impacts the spinal cord. The three components (brake, weight, and counterweight) are linked by a series of cables that are lifted in a pivot at the top of the system (Fig. 1A). Once the weight is dropped via the conducting tube, placed on top of the exposed spinal cord (Fig. 1E) and its tip touches the spinal cord, the braking system is released, quickly lifting the counterweight, and retrieving the weight from the impacted area. The dwell time is controlled and constant between experiments. The reproducibility of the impacts produced by this device was assessed prior to animal SCI. A testing platform, consisting of a hollow plastic cylinder covered by a tense silicone membrane, was used (Fig. 1F). A pressure transducer and an amplifier were coupled to this setup and a total of 20 impacts on the rubber membrane were run, from two different heights.

Spinal cord injury surgery and post-operative care

After deep anaesthesia induction, animals were submitted to a laminectomy between T7-T10 vertebrae, for exposure and visualization of the T8/T9 spinal segments.

For complete SCT, as in other studies (Chambel et al., 2022; Cruz et al., 2006a; Cruz et al., 2006b), thoracic vertebrae were exposed and a laminectomy was performed. The dura mater was pierced and the T8/T9 spinal cord was sectioned with a scalpel. A small piece of sterile haemostatic sponge was placed between the retracted ends of the cord to prevent bleeding. The surgical wound was closed in two layers and the animals were placed under post-surgical observation and care.

To perform mSCC and sSCC, after laminectomy, the animals were placed on the prepared platform for spinal impact. The caudal and dorsal vertebrae were firmly clamped with the forceps placed 1 cm from the desired impact zone. The system was adjusted so that the conducting cylinder for weight drop was very close to the exposed spinal cord (Fig. 1E). The brake system was activated to prevent accidental weight drop and the counterweight was suspended. After confirming the position of the animal and the conducting cylinder, the weight was placed in the conducting cylinder and dropped from 5 or 10 cm high, to respectively produce a mild or severe contusion of the spinal cord at T8/T9. Once the impact occurred, the weight immediately retracted. The wound was cleaned and sutured in two layers, followed by post-surgical care. Control animals underwent sham surgery in which the surgical wound was sutured after laminectomy without lesioning the spinal cord.

Post-surgical care for all groups consisted of subcutaneous administration of 1 mL of 5 % glycosylated saline after suturing, and antibiotics and analgesics for 8–10 days and 3–4 days, respectively. In SCI (SCT, mSCC and sSCC) animals, to avoid urinary retention, bladders were manually emptied by abdominal compression for 4 weeks. The voided volume was recorded every three days for later comparison.

Cystometries and terminal handling

To evaluate bladder reflex activity, animals (sham and SCI) were submitted to cystometries under anaesthesia 4 weeks after surgery. Following deep anaesthesia with subcutaneous urethane (1.2 g/Kg), a suprapubic skin incision was made, and muscle bundles separated for bladder exposure. A 21-gauge needle was inserted into the bladder dome, and sterile saline infused for 1 h at a rate of 6 ml/h. Bladder contractions were recorded by a pressure transducer connected to the needle. During this period, animals were placed on a heating plate to maintain body temperature at 37 °C. Cystometrograms were analysed using LabScribe software (World Precision Instruments, Hertfordshire, UK). Bladder contractions were disregarded if the amplitude was less than 5 cm H_2O.

Perfusion and tissue processing

After cystometry, animals were euthanized with an intraperitoneal injection of sodium pentobarbital (65 mg/Kg). Animals were perfused through the ascending aorta with calcium-free Tyrode's solution (0.12 M NaCl, 5.4 mM KCl, 1.6 mM MgCl2·H2O, 0.4 mM MgSO4·H2O, 1.2 mM

Table 1
Primary antibodies description.

Target	Dilution	Host specie	Manufacturer	Reference
CGRP	1:1000	Rabbit	Cell Signalling	14959
VAChT	1:1000	Rabbit	Synaptic Systems	sysy139103
TH	1:750	Rabbit	Abcam	ab137869
GAP43	1:1000	Rabbit	Abcam	ab16053
5HT	1:5000	Rabbit	Immunostar	ABIN617893
GFAP	1:2000	Mouse	Cell signalling	36705
Alexa 488 Anti-rabbit	1:1000	Donkey	Invitrogen	A21206
Alexa 568 Anti-mouse	1:1000	Donkey	Invitrogen	A10037

NaH2PO4·H2O, 5.5 mM glucose and 26.2 mM NaHCO3) followed by 4 % paraformaldehyde (PFA) in 0.1 M phosphate buffer. LUT organs (bladder and urethra), lumbosacral spinal segments (L5-S1) and the lesioned spinal site (T8/T9) were collected, post-fixed overnight in 4 % PFA and stored in 30 % sucrose in phosphate buffer with 0.1 % sodium azide for at least 24 h until further processing.

Collected bladders were sectioned in transversal, 20 µm thick slices, while urethras were cut into 12 µm thick longitudinal slices. The L5-S1 spinal cord was sectioned in transversal, 20 µm thick slices and the lesion site in longitudinal, 20 µm thick slices. Sections were obtained in a Leica cryostat (Leica, Famalicão, Portugal) and collected in Superfrost Plus slides. Slides were stored at −20° C until further processing.

Tissue morphology of the lesioned spinal cord

To analyze morphologic alterations at the lesion site, serial longitudinal sections of the lesion site (T8-T9 level) were left at room temperature for 24 h before staining with formol-thionine. Sections were incubated with acidic acetone for 5 min, followed by a period of 30 min in 10 % thionine in a 10 % formol solution. Sections were then washed in distilled water and mounted with Histomount mounting medium. Representative images obtained from three longitudinal sections, from each animal, in which the lesion site was identified in its entire length were investigated and collected in a Zeiss Axioscope 40 microscope using Leica LAS EZ v3.1.0 software (Leica Microsystems, Switzerland). The rostro-caudal extension of the lesioned spinal tissue was calculated with Image J. Results are presented as the percentage of the lesioned tissue in relation to the spinal cord area.

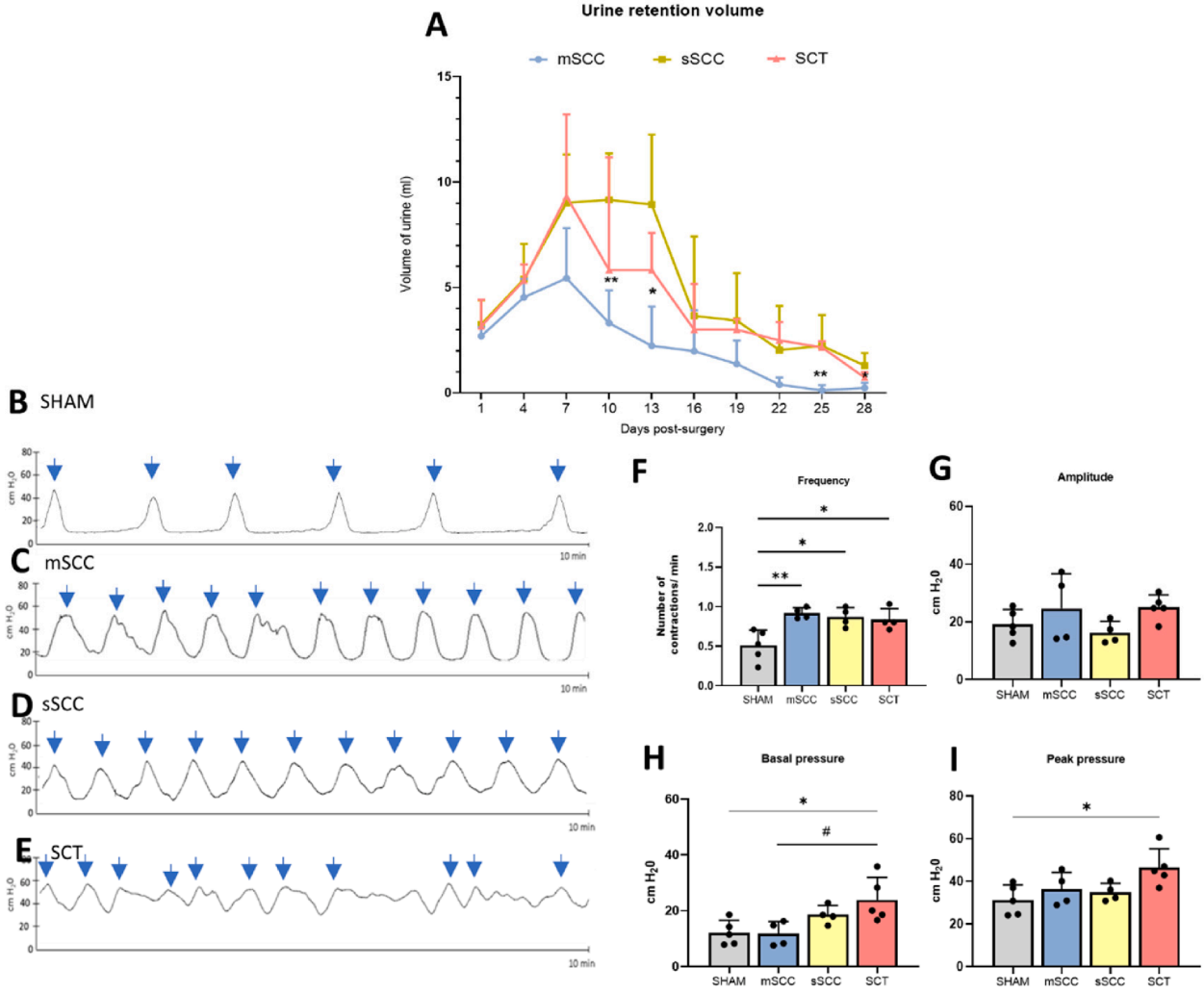

Fig. 2. Effects of experimental spinal cord contusion and transection on urinary function. (**A**) As SCI strongly affects urinary function and there is little or no bladder activity during the initial stages after spinal lesion, all animals were submitted to daily abdominal compression for urine removal. During this process, urine volumes were recorded. Data are presented on a 3-day interval basis. Values were recorded in mildly contused animals (mSCC), severely contused animals (sSCC), and fully transected animals (SCT). In SHAM animals, as the bladder was fully functional, urine volumes were unsignificant and therefore not recorded. Arrows indicate peaks of bladder reflex contractions.

Immunofluorescence analysis

Alternate sections from the bladder, urethra, lumbosacral spinal cord, and T8/T9 spinal cord tissue (lesion site) were thawed, washed in phosphate-buffered saline (PBS) and in PBS containing 0.3 % triton-X 100 (PBST), and blocked with 10 % normal horse serum (NHS) in PBST for 2 h. Tissue sections were incubated for 72 h at 4 °C with primary antibodies in 2 % PBST (Table 1). After several washes with PBST, sections were incubated with Alexa™ fluorochrome-labelled secondary antibody in 2 % PBST (Invitrogen – ThermoFisher Scientific, Porto, Portugal) for 1 h at room temperature. After subsequent washing, sections were mounted using an anti-fade mounting medium (Slowfade® Gold Life Technologies) and observed with an epifluorescence microscope (Axioimager Z1, Ziss Z1 from Zeiss) using the AxioVision 4.6 software.

Immunofluorescence labelling intensity was quantified by densitometry using Image J. For bladder tissue, five representative transverse sections of each animal were photographed in two distinct zones: the mucosa and the detrusor muscle. Only sections containing the bladder and urethral lumen were considered. In the case of urethral tissue, three longitudinal sections containing the urethral sphincter and in which the lumen was easily discernible were selected and photographed in three distinct zones: urethral mucosa, internal urethral sphincter (IUS) and external urethral sphincter (EUS). Results are presented as an average of the values measured in the posterior (close to the vagina) and anterior side of the urethral wall. In the case of spinal cord L5-S1 sections, at least 5 sections per segment (15 in total) were photographed in the following areas: dorsal horn, intermediolateral grey matter, central canal (lamina X) and ventral horn. Briefly, the mean level and standard deviation of background immunofluorescence were obtained in the region of interest without visible staining for each section analysed using ROI analysis. The threshold level for positive pixels was set at a value of 5 standard deviations above the mean background level. The mean percentage of staining was then calculated by delimiting the region of interest in each section and using ROI analysis (Chambel et al., 2022). In any case, whenever the staining procedure damaged the tissue under analysis and the adequate number of sections was not achieved, the animal was excluded. Nevertheless, even in groups in which we had to eliminate animals, the total number of sections per experimental group analysed was never less than 45 bladder sections, 10 urethral sections and 15 transverse lumbosacral spinal cord sections.

Statistics

Statistical analysis was conducted using GraphPad 8.2 software. The average impact force produced by the contusion device was compared using an unpaired *t*-test. The volumes of daily residual urine were analysed by a two-way ANOVA, followed by Tukey's Multiple comparison test. Data obtained in immunostaining analysis were first tested for normality (Shapiro-Wilk normality test). Normally distributed data were analysed using one-way ANOVA, followed by Tukey's Multiple comparison test. Abnormally distributed data were assessed by a non-parametric test (Krustal-Wallíśs test), followed by Dunńs multiple comparison test. In all cases, $p < 0.05$ was considered statistically significant. Data is presented as the mean ± standard deviation (SD). Further details can be found as supplementary information.

Results

Contusion and evaluation of the reproducibility of impact

To compare the effects of complete spinal cord transection and spinal contusion in LUT function, we used the well-established protocol of complete SCT (Cruz et al., 2006a; Cruz et al., 2006b; Oliveira et al., 2019) and a customised contusion device based on the weight-drop method, respectively. To test for potential variability between consecutive weight-drop trials, a hollow plastic cylinder covered by a silicone membrane was constructed and connected to a pressure transducer and an amplifier (Fig. 1F). The weight was dropped 20 times from 5 cm high and another 20 times from 10 cm high. The values recorded were, respectively, 11.24 ± 0.78 cm H_2O and 15.17 ± 1.260 cm H_2O (Fig. 1G), showing a statistically significant increase in impact intensity depending on the height the weight is dropped. The variation coefficients were 0.020 (drop from 5 cm high) and 0.018 (drop from 10 cm high).

Bladder dysfunction after spinal contusion and spinal transection

Every three days, urine volumes collected after daily abdominal compressions were recorded (Fig. 2A). Spinal intact animals were not submitted to daily abdominal compression, as there was no urinary retention following sham manipulation of the spinal cord. Previous unpublished observations from our group demonstrated that sham animals have a basal urine volume of 0.52 ± 0.18 mL. In SCI animals, the volume of urine collected on day 1 post-surgery was already high in all SCI groups. In the SCT group, urine volumes gradually increased, compared to the first day post-lesion, and reached the maximum average value of 9.00 ± 2.49 mL on day 7 post-SCT. From this time point onwards, urine volumes remained elevated but followed a descending trend. A similar variation was seen in the sSCC group on day 7 (7.80 ± 0.97 mL), with a decrease being observed only on day 13 post-lesion. In the mSCC group, the increase in urine volume was not as prominent, and the maximum urine volume (5.44 ± 0.57 mL) was recorded on day 7 post-contusion and decreased from that day onwards, reaching the average normal value 22 days after spinal injury. At timepoints of 10 ($p < 0.01$ versus sSCC), 13 ($p < 0.05$ versus sSCC) and 25 days after injury, the urine volume of mSCC animals was significantly lower than the urine volume of sSCC animals. At timepoint 25, mSCC animals presented significantly less urine than SCT animals ($p < 0.05$ versus SCT).

Cystometries under urethane anaesthesia demonstrated that spinal intact animals (SHAM group) presented normal bladder function (Fig. 2B). The frequency of bladder contractions, measured as the number of bladder contractions superior to 5 cm H_20 per min, was 0.51 ± 0.2 and the amplitude was 19.15 ± 5.16 cm H_2O (Fig. 2F, 2G). The basal bladder pressure was 12.10 ± 4.46 cm H_2O, and the maximum bladder pressure (peak pressure) was 31.25 ± 7.1 cm H_2O (Fig. 2H, 2I). Four weeks after the spinal lesion, several alterations in urodynamic parameters were observed, suggesting signs of bladder dysfunction in all SCI groups (Fig. 2C, 2D and 2E). We found no statistical differences between groups in the amplitude of bladder contractions (Fig. 2F). In contrast, the frequency of bladder reflex contractions increased in all SCI groups, when compared to sham animals (mSCC: 0.92 ± 0.07, *p< 0.05 versus SHAM; sSCC: 0.87 ± 0.12, $p < 0.05$ versus SHAM; SCT: 0.84 ± 0.14, *$p < 0.01$ versus SHAM; Fig. 2G). Basal pressure was only significantly increased in SCT rats (23.89 ± 8.04 cm H_2O, *$p < 0.05$ versus SHAM; #$p < 0.01$ versus mSCC), whereas mSCC (11.72 ± 4.43 cm H_2O) and sSCC rats (18.50 ± 3.35 cm H_2O) did not present significant differences in basal pressure, compared to SHAM animals (Fig. 2H). Peak pressure followed the same pattern, with only the SCT group presenting significantly different values in comparison to SHAM animals (mSCC: 36.39 cm H_2O±7.85; sSCC: 34.84 ± 4.2 cm H_2O; SCT: 46.48 ± 8.8 cm H_2O, *$p < 0.05$ versus SHAM) (Fig. 2I).

Bladder contractility was assessed by cystometry under urethane anaesthesia, in intact (**B-SHAM**), mild contused (**C-mSCC**), severe contused (**D-sSCC**) and fully transected animals (**E-SCT**). Urodynamic parameters of the amplitude (**F**), frequency of bladder reflex contractions (**G**), Basal pressure (**H**), and peak pressure (**I**) of the overall experimental groups. The residual urine volumes were analysed by a two-way ANOVA followed by Turkeýs multiple comparison test. The urodynamic data was tested by a one-way ANOVA, followed by Turkeýs multiple comparison test (*$p < 0.05$, **$p < 0.01$ versus SHAM; #$p < 0.05$ versus mSCC).

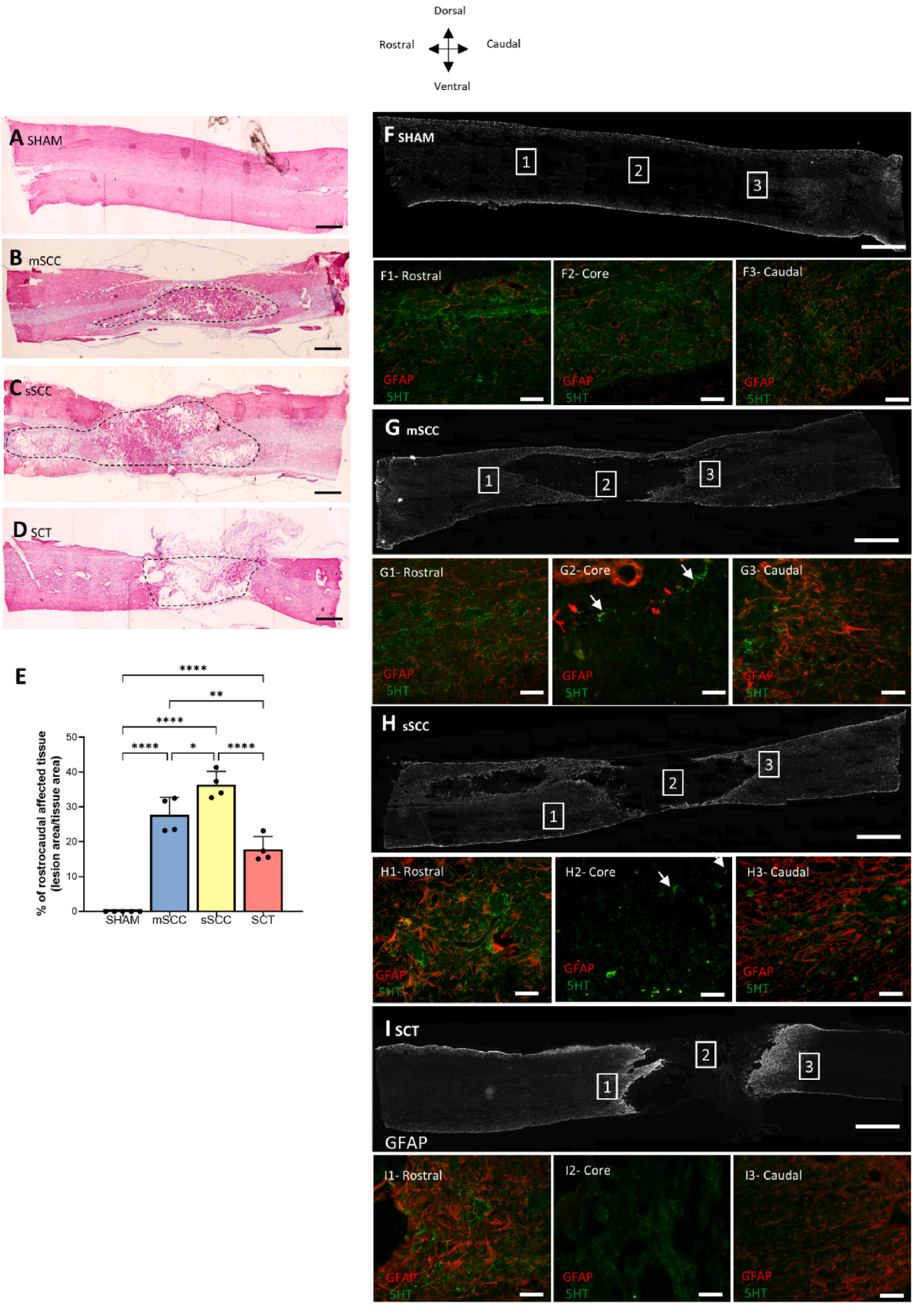

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Fig. 3. Effects of different types of spinal cord injury on the histology of the lesioned area. Representative images of formol-thionin stained lesion site tissue. (**A – SHAM**) In sections from sham-manipulated animals, there was no evidence of neuronal damage at the laminectomy site. (**B – mSCC**) Sections from animals submitted to mild contusion (mSCC) presented a compacted lesion area, with well-defined borders and signs of preserved tissue. (**D – sSCC**) In sections from animals submitted to severe contusion (sSCC) the lesion area was more widespread, with poorly defined borders that were accompanied by severe neuronal loss. (**D – SCT**) In sections from animals submitted to complete spinal cord transection (SCT), there was total disruption of spinal tissue between the caudal and rostral ends of the injury site. Scale bars equal 50 μm. (**E**) Quantification of the area of lesioned tissue was performed using image J. Data were compared using One-Way ANOVA followed by Tukey's multiple comparison test (***$p < 0.005$; ****$p < 0.0001$). The completeness of the lesion existence of spared axons was analysed by immunofluorescence against Glial fibrillary acidic protein (GFAP) and serotonin (5-HT). (**F**) GFAP immunostaining was very faint in sections from SHAM while 5-HT immunoreactive profiles were evident in all the extension of the sections. (**G**) In sections from mSCC animals it was possible to identify the scar tissue, surrounded by rostral and caudal GFAP immunostaining with some 5-HT positive profiles in the lesion core (**G1**) as well as in more caudal regions (**G2**). (**H**) In sections from sSCC animals it was also possible to identify the scar tissue, with strong surrounding GFAP immunostaining and fewer 5-HT positive fibres in the lesion core (**H1**) and in more caudal regions of the analysed sections (**H2**). (**I**)The scar tissue was evident in sections from SCT animals, with intense GFAP immunlabelling and no signs of 5-HT immunoreactive fibres in the lesion core (**I2**) and more caudal regions (**I3**).

Tissue damage at the lesion site

To evaluate the degree of damage at the thoracic spinal cord produced by SCT, mSCC and sSCC, longitudinal spinal sections of the injury site were stained with formol-thionin, a classical staining method to study the cytoarchitecture of neuronal tissue (Donovick, 1974), that we have previously used to analyse SCI (Oliveira et al., 2019) (Fig. 3). In sham-manipulated animals, neuronal tissue was intact (Fig. 3A). In contrast, spinal tissue damage was evident in samples from all SCI animals (****$p < 0.0001$ versus SHAM). In the areas of injury, staining allowed the identification of cavities and tissue integrity. In animals submitted to contusion, cavities were small, randomly distributed, with some preservation of grey matter, particularly in sections from mSCC animals. In sections from SCT animals, the cavities were larger and connected, with a complete interruption of the grey matter (Fig. 3A-D).

In groups submitted to spinal contusion, the area of damaged tissue was evident (Fig. 3B, C and D; ****$p < 0.0001$ versus SHAM, for both mSCC and sSCC) and the rostro-caudal extension was directly proportional to the severity of the contusion. In the sSCC group, the percentage of lesioned tissue was 36.36 % ± 3.87 of the total area, significantly higher than what was observed in mSCC rats, in which the lesioned tissue represented 27.76 % ± 5.04 of the total area analysed (***$p < 0.001$ versus sSCC animals). In the SCT group, the lesioned area was restricted to the vicinity of severed ends of the cord, as the centre was occupied by scar tissue. The rostro-caudal extension was smaller than mSCC and sSCC and represented 17.8 % ± 3.7 of the total area analysed (Fig. 3D and 5E; ****$p < 0.0001$ versus SHAM; *$p < 0.05$ versus mSCC; ****$p < 0.0001$ versus sSCC).

To evaluate the completeness of the lesion and axonal sparing, longitudinal spinal sections of the injury site were immunostained against GFAP and 5HT, respectively. SHAM animals presented very faint GFAP immunolabelling and had a normal pattern of 5-HT positive axons, visible along the entire length of the section (Fig. 3F). Both mSCC and sSCC animals displayed well-defined glial scar borders, with evident GFAP immunostaining surrounding the lesion core (Fig. 3G and H). In these sections, staining against 5-HT showed that conserved axons are present in the borders of the lesion, with some remaining even in its core (Fig. 3G2, 3H2). In some cases, 5-HT positive fibres could be observed caudal to the lesion core, more frequent and visible in sections from mSCC (Fig. 3G3) rather than sSCC rats (Fig. 3G4). In animals submitted to complete transection, the glial scar was visibly more intense (Fig. 3I), and no signs of spared 5-HT immunolabelled profiles were observed in the lesion core (Fig. 3I2) or in locations caudal to injury (Fig. 3I3).

Neuronal sprouting at the bladder and lower urinary tract after spinal cord contusion and transection

It is known that SCT courses with increased axonal sprouting at the lumbosacral spinal cord and LUT (Chambel et al., 2022; Ferreira et al., 2022; Oliveira et al., 2019) but it was not clear if the same would happen after spinal contusion at thoracic segments. As before, growth-associated protein-43 (GAP43) expression was used as a marker of axonal sprouting (Benowitz and Routtenberg, 1997).

In the bladder, we found that GAP43 was absent in the mucosa of all SCI animals, compared to sham animals (**$p < 0.01$ versus SHAM) (Fig. 4A, D, E, F and G). In the detrusor muscle, the decreased expression of GAP43 was less evident, being only observed in sections from SCT animals when compared to sham animals (*$p < 0.05$ versus SHAM) (Fig. 4A, H, I, J and K). In the urethral mucosa, GAP43 immunostaining was abundant in sham animals, but strongly decreased in all injured animals, particularly in mSCC animals (*$p < 0.05$, **$p < 0.01$ versus SHAM; Fig. 4B, L, M, N and O). The urethral sphincter followed the same tendency, with the internal sphincter of all injured animals showing similar patterns of GAP43 downregulation, compared to sham animals (*$p < 0.05$, **$p < 0.01$ versus SHAM; Fig. 4B, L, M, N and O). Likewise, GAP43 expression was elevated in the striated muscle of the external urethral sphincter in sham animals and decreased after spinal injury, particularly in contusioned animals, although a tendency for a decrease in SCT animals was also observed (*$p < 0.05$ versus SHAM; Fig. 4P, Q, R, and S).

GAP43 expression was also evaluated at the L5-S1 spinal cord level, the spinal segments receiving sensory input generated in the LUT (Morgan et al., 1981). We found GAP43 immunoreactivity in lamina I-II of the dorsal horn (Lam I-II), in the intermediolateral grey matter (IML), and laminae X (Lam X), the pivotal areas involved in LUT sensory processing and autonomic regulation. In lamina I-II, we found an upregulation of GAP43 levels, particularly in animals submitted to spinal contusion (**$p < 0.01$, ****$p < 0.0001$ versus SHAM Fig. 4C, T, U, V and W). In sections from SCT animals, changes in GAP43 expression did not reach statistical difference, compared to sham animals, despite a tendency to increase, but did reach significance in comparison with mSCC (###$p < 0.001$ versus mSCC). In the IML, all SCI groups presented the same pattern of increased GAP43 expression, compared to sham animals (*$p < 0.05$, **$p < 0.01$ versus SHAM; Fig. 4C, X, Y Z and A1). In lamina X, no statistically significant differences between experimental groups were found (Fig. 4C, A2, A3, A4 and A5).

Expression of markers of sensory and autonomic tracts in the lower urinary tract and lumbosacral cord after spinal contusion and transection

Sensory innervation

To examine changes in sensory innervation, we studied the expression of calcitonin-gene-related peptide (CGRP), a neuropeptide present in peptidergic sensory fibres (Snider and McMahon, 1998) and widely expressed by bladder afferents (Avelino et al., 2002). Analysis of CGRP immunostaining showed that this sensory marker was absent in the bladder mucosa of all SCI groups when compared to sham animals (****$p < 0.0001$ versus SHAM) (Fig. 5A, D, E, F and G). In the detrusor muscle, there was a reduction in CGRP levels in SCI animals (**$p < 0.01$ versus SHAM; Fig. 5A, H, I, J and K).

In the urethra, there was also an evident loss of CGRP expression in all SCI groups (**$p < 0.01$; ***$p < 0.001$ versus SHAM; Fig. 5B, L, M, N and O). In the urethral sphincters, we found decreased CGRP levels in both the IUS and EUS. In the IUS, the pattern of CGRP denervation was

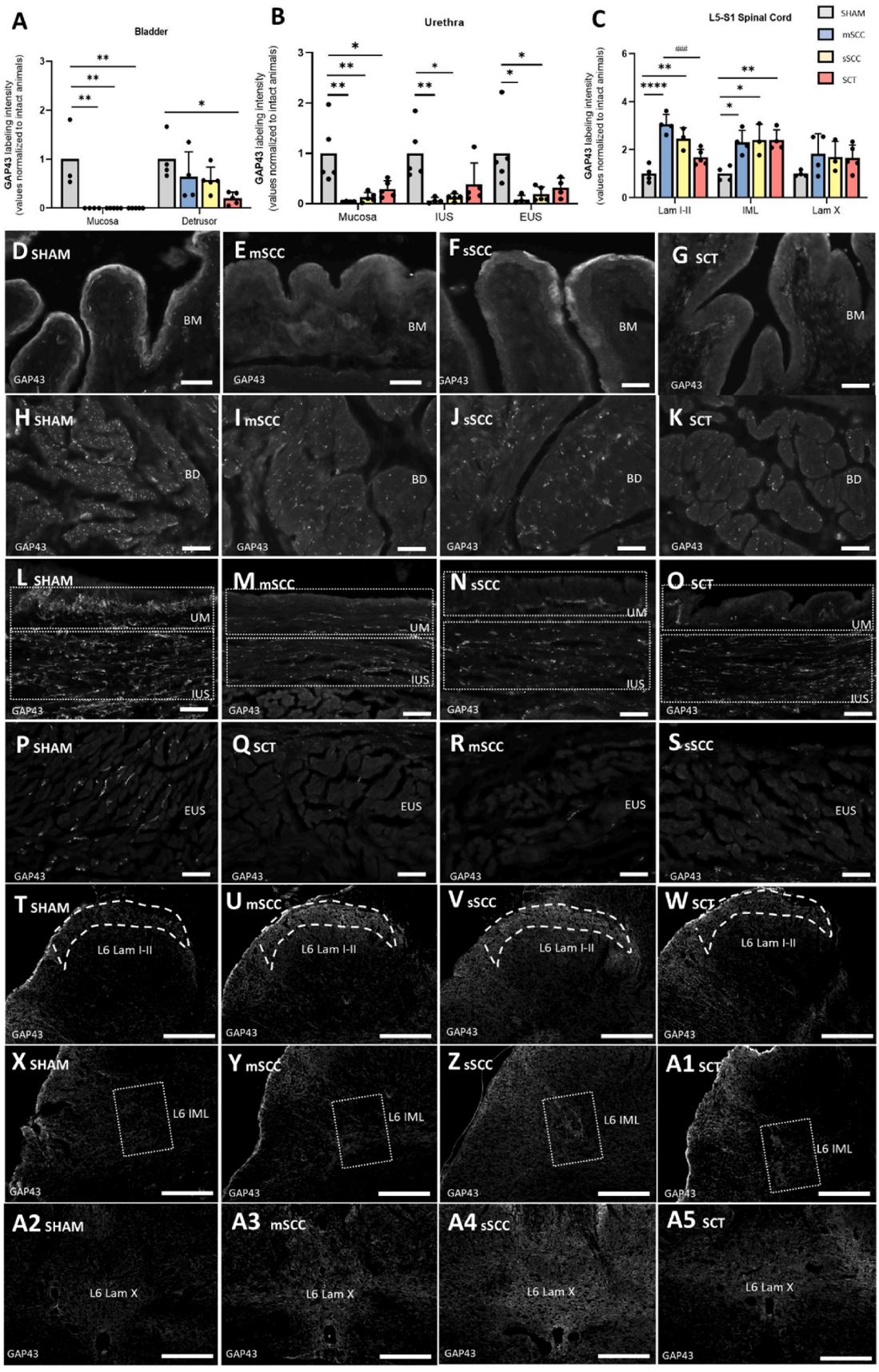

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Fig. 4. Assessment of neuronal sprouting after mild and severe spinal cord contusion and spinal cord transection. Axonal sprouting was accessed by GAP43 immunostaining, a well-established marker of neuronal growth. Expression of GAP43 was quantified in the bladder (**A**), urethra (**B**) and L5-S1 spinal cord segments (**C**). In the bladder, GAP43 levels were studied in the bladder mucosa of SHAM (**D**), mSCC (**E**), sSCC (**F**) and SCT animals (**G**), and a marked decrease was observed in sections from SCI animals. The analysis of the bladder detrusor of SHAM (**H**), mSCC (**I**), sSCC (**J**) and SCT animals (**K**) showed the same tendency of GAP43 downregulation. In the urethra, three zones were analysed: urethral mucosa and the internal (IUS) and external (EUS) sphincters. In the mucosa and IUS of all groups, SHAM (**L**), mSCC (**M**), sSCC (**N**) and SCT animals (**O**), GAP43 was also downregulated in animals submitted to spinal contusion but not transection. In the EUS, in comparison with SHAM animals (**P**), GAP43 was also decreased in sections from mSCC (**Q**), sSCC (**R**) but not from SCT rats (**S**). GAP43 expression was also analysed in the lumbosacral spinal cord. The observation of lamina I-II areas of SHAM (**T**), mSCC (**U**), sSCC (**V**) and SCT animals (**X**) showed that mSCC animals present higher GAP43 levels than the remaining SCI groups. In the intermediolateral nucleus (IML) of SHAM (**Z**), mSCC (**A1**), sSCC (**A2**) and SCT animals (**A3**), we observed increased sprouting in all injured groups, irrespectively of the SCI model. In the Laminae X of intact (**A3**), mSCC (**A4**), sSCC (**A5**) and SCT animals (**A6**), no differences were found. Normally distributed data was compared using One-Way ANOVA followed by Tukey's multiple comparison tests. Non-normally distributed data was compared using Krustal Wallis test followed by Dunńs multiple comparison test (*$p < 0.05$, **$p < 0.01$; **** $p < 0.0001$ versus SHAM; ##$p < 0.01$ versus mSCC). Scale bars equal 50 μm.

similar to that observed in the mucosa (*$p < 0.05$ versus SHAM; Fig. 5B, L, M, N and O). In the striated muscle of the EUS, decreased expression of CGRP was observed only in mSCC and SCT animals, when compared to SHAM (*$p < 0.05$, **$p < 0.01$ versus SHAM; Fig. 5B, P, Q, R and S). The expression of CGRP was also investigated in the L5-S1 spinal segments of SHAM and SCI animals. In the dorsal horn (Lam I-II), we found that CGRP levels were significantly increased in animals submitted to mSCC (*$p < 0.05$ versus SHAM; Fig. 5C, T, U, V, and W). CGRP content in SCT animals was significantly reduced when compared with mSCC (#$p < 0.05$ versus mSCC). In the IML, CGRP levels were also increased, reaching statistical significance in mSCC animals when compared with SHAM (**$p < 0.01$ versus SHAM) and the other SCI animals (#$p < 0.05$ versus sSCC; #$p < 0.05$ versus SCT) (Fig. 5C, X, Y, Z and A1). No alteration was found in the Lam X area (Fig. 5C, A2, A3, A4 and A5).

Cholinergic innervation: Expression of vesicular acetylcholine transporter (VAChT)

To investigate cholinergic innervation, the expression of vesicular acetylcholine transporter (VAChT) was studied in the LUT and lumbosacral cord. In the bladder, like CGRP, VAChT expression was absent in the bladder mucosa of SCI animals (Fig. 6A, D, E, F and G) when compared to SHAM animals (Fig. 6A and D), irrespective of the type of spinal injury. Likewise, we also found a dramatic reduction in VAChT levels in the detrusor muscle of SCI animals, particularly in sSCC and SCT (*$p < 0.05$; versus SHAM; Fig. 6A, H, I, J and K). In the urethra, there were also changes in VAChT levels. A marked reduction in the expression of this marker was found after spinal contusion or transection when compared to sham animals (****$p < 0.0001$ versus SHAM; Fig. 6B, L, M, N and O). The urethral sphincter was affected, with evidence of downregulation of VAChT expression in both the IUS and EUS (****$p < 0.0001$ versus SHAM, **$p < 0.01$ versus SHAM; Fig. 6B, L, M, N and O; P, Q, R and S).

In the L5-S1 spinal cord segments, the presence of VAChT was also analysed. VAChT was absent from laminae I-II but present in the ILG, lamina X and ventral horn of spinal intact animals. In the IML, VAChT expression was similar between SHAM and mSCC animals but decreased after sSCC and SCT (*$p < 0.05$, ***$p < 0.001$ versus SHAM; mSCC; Fig. 6C, T, U, V and W). The SCT group also presented decreased VAChT immunostaining, compared to mSCC (##$p < 0.01$ versus mSCC), being the group that presented the most evident reduction in VAChT expression. No alterations were found in the Lam X area (Fig. 6XY, Z and A1). In the ventral horn, VAChT expression was only reduced in SCT animals when compared to mSCC (#$p < 0.05$ versus mSCC; Fig. 6A2, A3, A4 and A5).

Noradrenergic innervation: Expression of tyrosine hydroxylase (TH)

Noradrenergic innervation was assessed by investigating tyrosine hydroxylase (TH) expression. In the bladder, no TH-positive profiles were observed, with only a reduced number of fibres present in the vicinity of small-calibre blood vessels (Fig. 7C). In the urethra, the presence of noradrenergic fibres was detected in the urethral sphincter, but not in the mucosa. In the IUS, TH-positive fibres were decreased after SCI, particularly in mSCC animals (*$p < 0.05$ versus SHAM), while in sSCC and SCT animals, downregulation of TH expression did not reach statistical significance (Fig. 7A, D, E, F and G). The same tendency was found in the EUS, where the reduction in TH expression was statistically significant in mSCC and sSCC (*$p < 0.05$ versus SHAM), but not in SCT animals (Fig. 7A, H, I, J and K).

At the lumbosacral spinal cord level, expression of TH was found in laminae X, where levels of this noradrenergic marker were reduced after SCI, irrespective of the type of insult (**$p < 0.01$, ***$p < 0.001$ versus SHAM; Fig. 7B, L, M, N and O).

Discussion

A better understanding of the pathophysiological mechanisms of SCI-induced LUT dysfunction is critical to promote advances in medical treatment. In this context, the use of animal models has been essential, as they allow the understanding of complex mechanisms of SCI and how they may affect LUT activity (Ferreira et al., 2023; Sharif-Alhoseini et al., 2017). The most commonly used experimental SCI protocol has been the complete SCT, a highly reproducible experimental protocol, but with a debatable translational value, as the majority of SCI result from contusion injuries. Therefore, it is important to clarify the similarities and differences in LUT function and reorganization of neuronal pathways after spinal contusion and transection. This has been a matter under investigation for some years (Breyer et al., 2017; Mitsui et al., 2014; Pikov et al., 1998) and here we expanded previous observations, comparing spinal damage, LUT function, and changes in the LUT and lumbosacral spinal cord after mild and severe contusion and transection at T8/T9 spinal segment.

Design of a new contusion method: model advantages and validation

In this study, we used a simple-to-use and affordable set-up to produce spinal cord contusion. This setup is an adaptation of the classical weight-drop method used by others (Lima et al., 2021). The weight-drop method was the first described method to experimentally produce a spinal cord contusion in rodents (Allen, 1911). More recently, sophisticated automated impactors have been introduced, allowing the production of contusive spinal injuries in a controlled and reproducible manner (Rabchevsky et al., 2003; Sharif-Alhoseini et al., 2017). Yet, these systems are costly and involve maintenance costs, which may preclude their widespread use. Here, we produced a simple and inexpensive alternative based on the classical weight-drop method, which includes a braking component that ensures that the impactor tip is immediately retracted following impact.

An important concern when using our setup is its reproducibility. Although we did not have access to a force transducer, our results demonstrated a correlation between the height of the weight drop and the force produced on a silicone membrane, with low deviations between trials. Thus, the device used in this study can be used to produce reproducible spinal cord contusions of different severities by adjusting the height of the weight drop. This was confirmed by histological

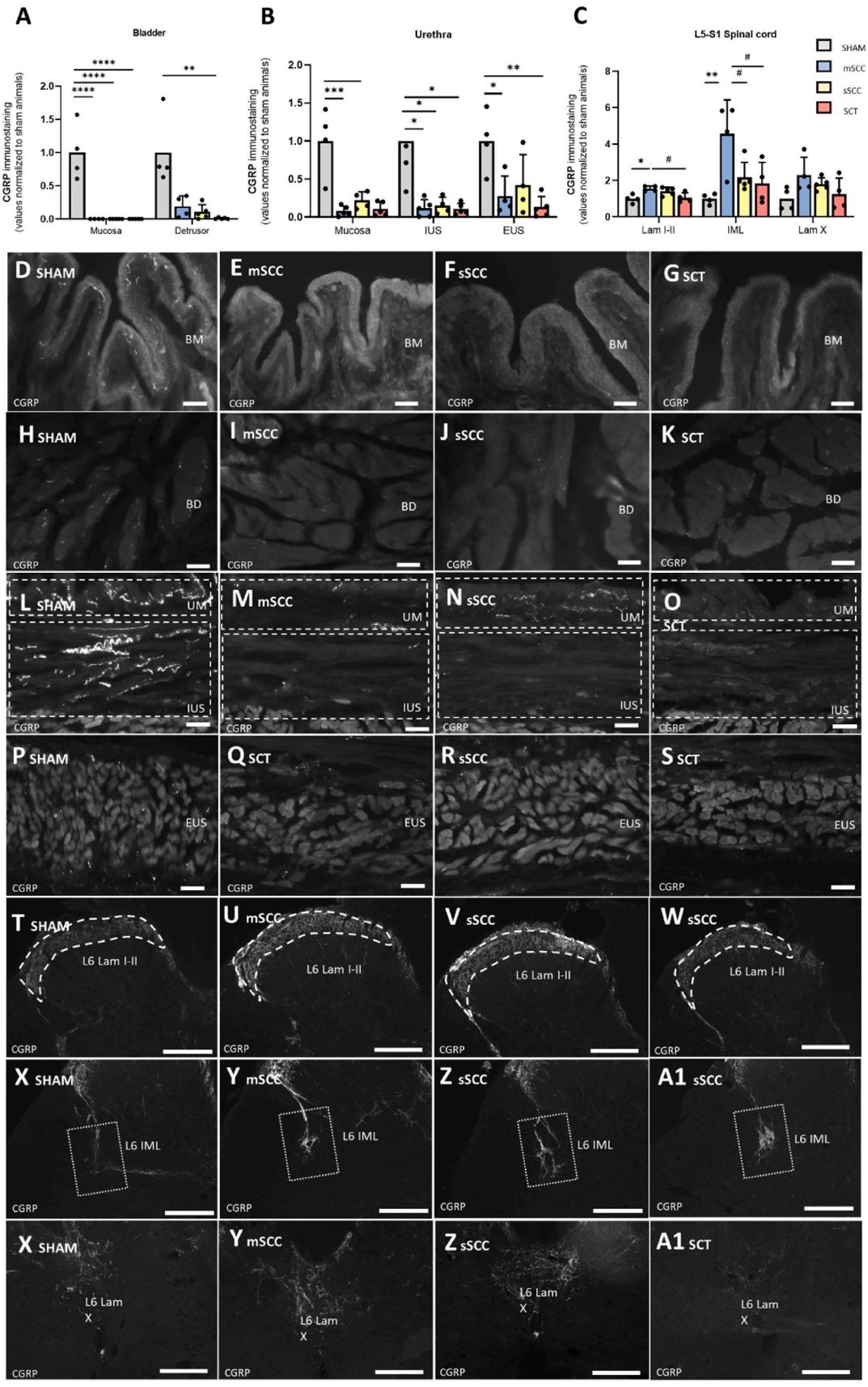

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Fig. 5. Calcitonin Gene-related peptide (CGRP) expression in the LUT and lumbosacral spinal cord after SCI. The distribution of sensory fibres was assessed by immunostaining against CGRP, a well-established marker of peptidergic afferents. The expression of CGRP was quantified in the bladder (**A**), urethra (**B**) and L5-S1 spinal cord segments (**C**). In the bladder, the mucosa of SHAM (**D**), mSCC (**E**), sSCC (**F**) and SCT animals (**G**) was analysed and showed absence of CGRP after SCI, irrespective of the type of injury. Likewise, in the bladder detrusor of SHAM (**H**), mSCC (**I**), sSCC (**J**) and SCT animals (**K**) there was also CGRP downregulation. In the urethra, three zones were analysed: the urethral mucosa and the internal (IUS) and external (EUS) sphincters. In the mucosa and IUS of SHAM (**L**), mSCC (**M**), sSCC (**N**) and SCT animals (**O**), CGRP was downregulated in all SCI groups. The EUS was also assessed in SHAM (**P**), mSCC (**Q**), sSCC (**R**) and SCT animals (**S**), where significant loss of CGRP immunostaining was only seen in sections from mSCC and SCT animals. CGRP was also evaluated at the lumbosacral spinal cord. The analysis of lamina I-II of SHAM (**T**), mSCC (**U**), sSCC (**V**) and SCT animals (**X**) showed that mSCC animals presented significantly higher levels of CGRP immunostaining, compared to SCI models. In the intermediolateral nucleus (IML) of SHAM (**Z**), mSCC (**A1**), sSCC (**A2**) and SCT animals (**A3**) CGRP upregulation was also observed. No differences were found in the Laminae X of SHAM (**A3**), mSCC (**A4**), sSCC (**A5**) and SCT animals (**A6**). Normally distributed data was compared using One-Way ANOVA followed by Tukey's multiple comparison tests. Non-normally distributed data was compared using Krustal Wallis test followed by Dunńs multiple comparison test (*$p < 0.05$, **$p < 0.01$; ***$p < 0.001$, **** $p < 0.0001$ versus SHAM; # $p < 0.05$ versus mSCC). Scale bars equal 50 μm.

analysis of the injured spinal tissue, as well as by analysing GFAP and 5-HT immunostaining. Accordingly, we observed an extensive area of tissue damage after spinal contusion, which was larger and more evident in animals submitted to a 10 cm-high weight drop (severe spinal contusion), compared to rats on which the weight was dropped from 5 cm high (mild spinal contusion). Interestingly, the area of damaged tissue was more constrained, with a more limited rostrocaudal distribution of lesioned tissue, in animals submitted to spinal transection, reflecting the limited injury induced by restricted insertion of the scalpel blade. However, a more restricted spread of damage rostrocaudally does not necessarily mean a less severe injury. In fact, analysis of GFAP and 5-HT immunolabelling showed axonal sparing after contusion, particularly after mild contusion, with some positive fibres in the core of the lesioned tissue and caudally to the injury. In SCT, these 5-HT immunoreactive fibres were only present in the rostral spinal cord in relation to the injury, and absent in the rest of the sections, indicating that SCT produces a complete and more severe injury of the cord.

Effect of different SCI models on bladder function

Spinal lesion is followed by spinal shock, during which the bladder has little or no reflex activity (Anderson et al., 2023; Bywater et al., 2018). During this period, SCI animals need to be submitted to daily abdominal compression for urine removal. Residual urine volumes were recorded every three days, and we observed an increase in urine volume in all SCI animals during the first week post-spinal trauma, which was reduced upon the emergence of spontaneous voiding. While a reduction in urine volume was found from post-injury day 7 onwards in SCT and mSCC animals, in sSCC this was only observed from post-injury day 13. This suggests that the development of spontaneous voiding is affected by the area of damaged tissue. Indeed, in sSCC animals the area of lesioned tissue seen after severe contusion was extensive, affecting more rostral and caudal segments beyond the injury site. This larger area of damage, not seen in SCT or mSCC animals, likely delays tissue healing and precludes neural recovery, resulting in more prolonged spinal shock.

Bladder function was also studied by cystometry in sham and SCI animals. As others (Breyer et al., 2017; Mitsui et al., 2014), we did not find any major differences between SCI animals and only observed higher basal and peak pressures in SCT animals in comparison to spinal intact rats. The lack of significant differences in bladder function after mild and severe spinal contusion and spinal transection suggest that, despite different degrees of damage to the spinal cord, injuries resulted in similar functional impairment of the lower urinary tract (Pikov et al., 1998). While others have performed awake cystometries (Breyer et al., 2017; Mitsui et al., 2014), in this study urethane was used, which could have affected our observations. This anaesthetic remains a suitable option to study bladder reflex activity, as others produce a more detrimental effect on bladder function (Aizawa and Fujita, 2023; Yaksh et al., 1986). It should, however, be recalled that urethane affects urethral function, leading to increased sphincter resistance and residual volume (Chang and Havton, 2008). This effect of urethane is likely more pronounced in SCT rats (Yoshiyama et al., 2013), which lack supraspinal control over bladder function, leading to higher basal and peak pressures than in sham rats.

Effect of different SCI models on LUT innervation

Neuronal sprouting is a key mechanism to NDO emergence in response to the loss of bulbospinal input, leading to the strengthening of spinal synapses and the establishment of new ones at the lumbosacral spinal cord, accompanied by changes in afferent excitability (Shimizu et al., 2023; Wada et al., 2022). Axonal sprouting can be assessed by analysing the expression of GAP43, a well-established marker of neuronal sprouting (Benowitz and Routtenberg, 1997). In the LUT, GAP43 was absent in the bladder and urethral mucosa of SCI animals, irrespective of the type of injury, consistent with a lack of difference in bladder dysfunction. In the detrusor, GAP43 was significantly decreased in SCT animals, with a non-significant tendency for reduction in animals submitted to spinal contusion. While this may suggest that there is no remodelling of detrusor innervation after spinal injury, this is unlikely as the bladder muscle undergoes major tissue remodelling after SCI (Haferkamp et al., 2003; Johnston et al., 2012), resulting in bladder hypertrophy (Mimata et al., 1993; Nagatomi et al., 2005) and more disperse innervation, which complicates the assessment of GAP43 expression. In the urethral sphincter, GAP43 was also reduced in the IUS and EUS of mSCC and sSCC, but not after SCT. This is in line with our previous study which has also analysed GAP43 levels in urethral sections from SCT rats at the same time point (Ferreira et al., 2022). While in that study we did not separately examine GAP43 in different zones of the urethral wall, we also did not find any significant differences in the IUS and EUS. Overall, our results indicate that there is neuroplasticity in the LUT, particularly in the mucosa of the bladder and urethra. This reduction in GAP43 levels may also reflect LUT denervation as seen in human (Drake et al., 2000) and rodent SCI (Johnston et al. 2012).

Changes in sensory innervation of the bladder and urethra were investigated by analysing CGRP levels, a neuropeptide abundantly expressed in bladder afferents (Avelino et al., 2002; de Rijk et al., 2024). We found a marked decrease in CGRP levels in all layers of the bladder and urethral walls. The reasons for this sensory denervation may only be speculated at present but it may arise during spinal shock. Indeed, sensory fibres are highly dependent on Nerve Growth Factor (NGF) (Cruz, 2014; Denk et al., 2017), which is produced by LUT smooth muscle cells during contraction (Clemow et al., 2000; Persson et al., 1997). As in spinal shock there is little or no bladder reflex activity (Anderson et al., 2023; Bywater et al., 2018) and loss of smooth muscle cells in the urethra after SCI (Ferreira et al., 2022), this may cause a reduction in NGF, which may lead to sensory denervation of the LUT.

Cholinergic innervation was evaluated by investigating the expression of VAChT. In the bladder mucosa and detrusor muscle, we found a similar reduction in VAChT levels in all SCI animals, irrespective of the type of injury and in agreement with previous studies (Breyer et al., 2017; Johnston et al., 2012; Takahara et al., 2007). In the urethra, VAChT immunoreactivity was also reduced in the mucosa, IUS and EUS. Loss of VAChT immunoreactivity suggests dysfunction of cholinergic neurotransmission in this organ (Roy and Green, 2019; Takahara et al., 2007). Moreover, the recovery of the expression of this marker may be

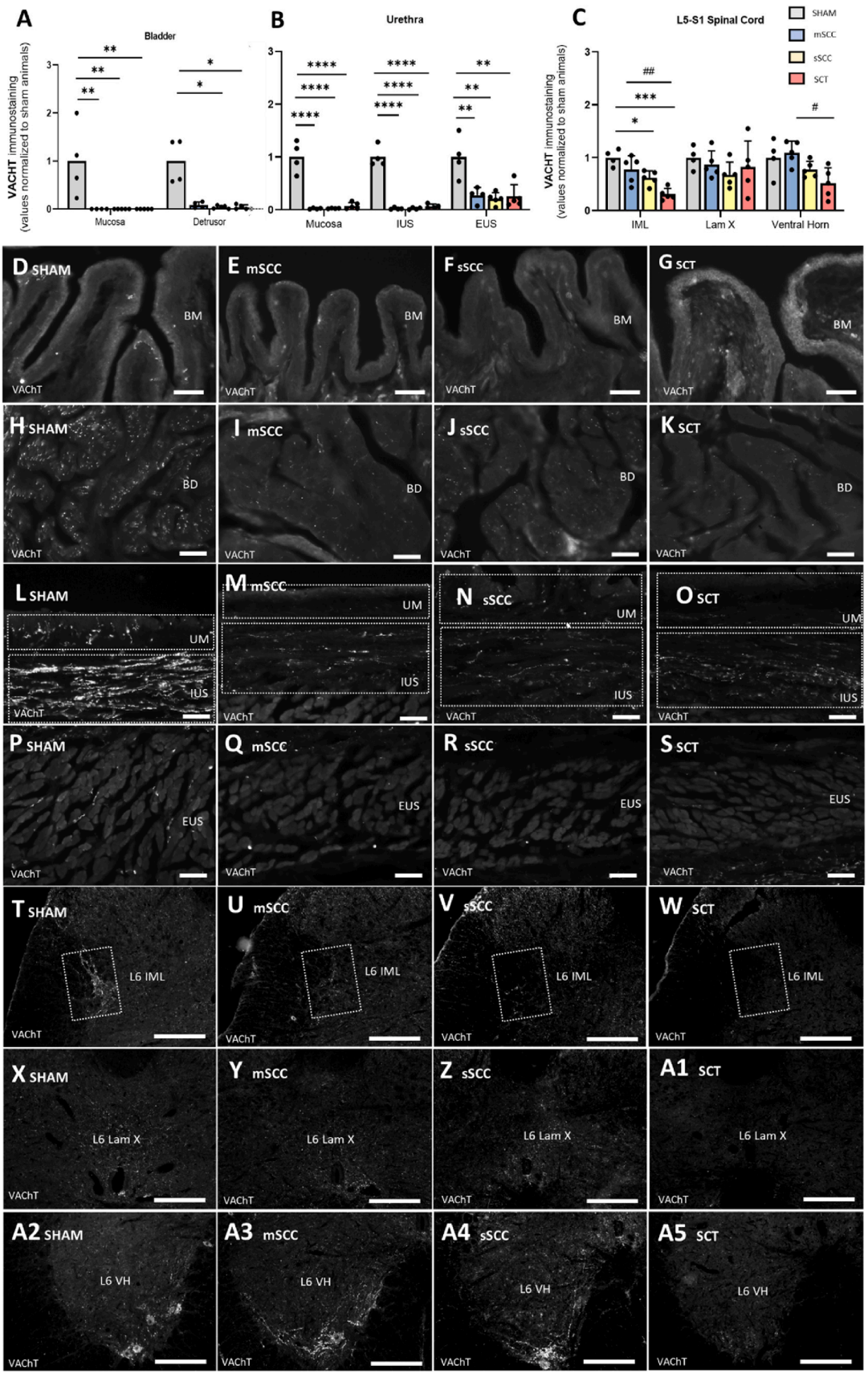

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Fig. 6. Expression of Vesicular Transporter of Acetylcholine (VAChT) after mild and severe spinal cord contusion and spinal cord transection. Cholinergic innervation was analysed by immunostaining against VAChT, a well-established marker of cholinergic fibres. The expression of CGRP was quantified in the bladder (**A**), urethra (**B**) and L5-S1 spinal cord segments (**C**). In the bladder, we found a significant decrease in VAChT levels in the bladder mucosa of SHAM (**D**), mSCC (**E**), sSCC (**F**) and SCT animals (**G**). VAChT was also decreased in the detrusor of SHAM (**H**), mSCC (**I**), sSCC (**J**) and SCT animals (**K**). In the urethral mucosa and the internal urethral sphincter (IUS) of SHAM (**L**), mSCC (**M**), sSCC (**N**) and SCT animals (**O**), VAChT levels were also decreased, irrespective of the type of SCI. VAChT downregulation was also observed in the EUS of SHAM (**P**), mSCC (**Q**), sSCC (**R**) and SCT animals (**S**). Levels of VAChT were also studied in the lumbosacral spinal cord, which were found in the IML, laminae X and ventral horn. The analysis of the intermediolateral nucleus (IML) of SHAM (**T**), mSCC (**U**), sSCC (**V**) and SCT animals (**W**) showed that loss of VAChT immunostaining was only seen in sections from sSCC and SCT animals. No differences were found in the laminae X of SHAM (**X**), mSCC (**Y**), sSCC (**Z**) and SCT animals (**A1**). A similar response was observed in the ventral horn of SHAM (**A2**), mSCC (**A3**), sSCC (**A4**) and SCT animals (**A5**). Normally distributed data was compared using One-Way ANOVA followed by Tukey's multiple comparison tests. Non-normal data was compared using Krustal Wallis test followed by Dunńs multiple comparison test. Scale bars equal 50 μm.

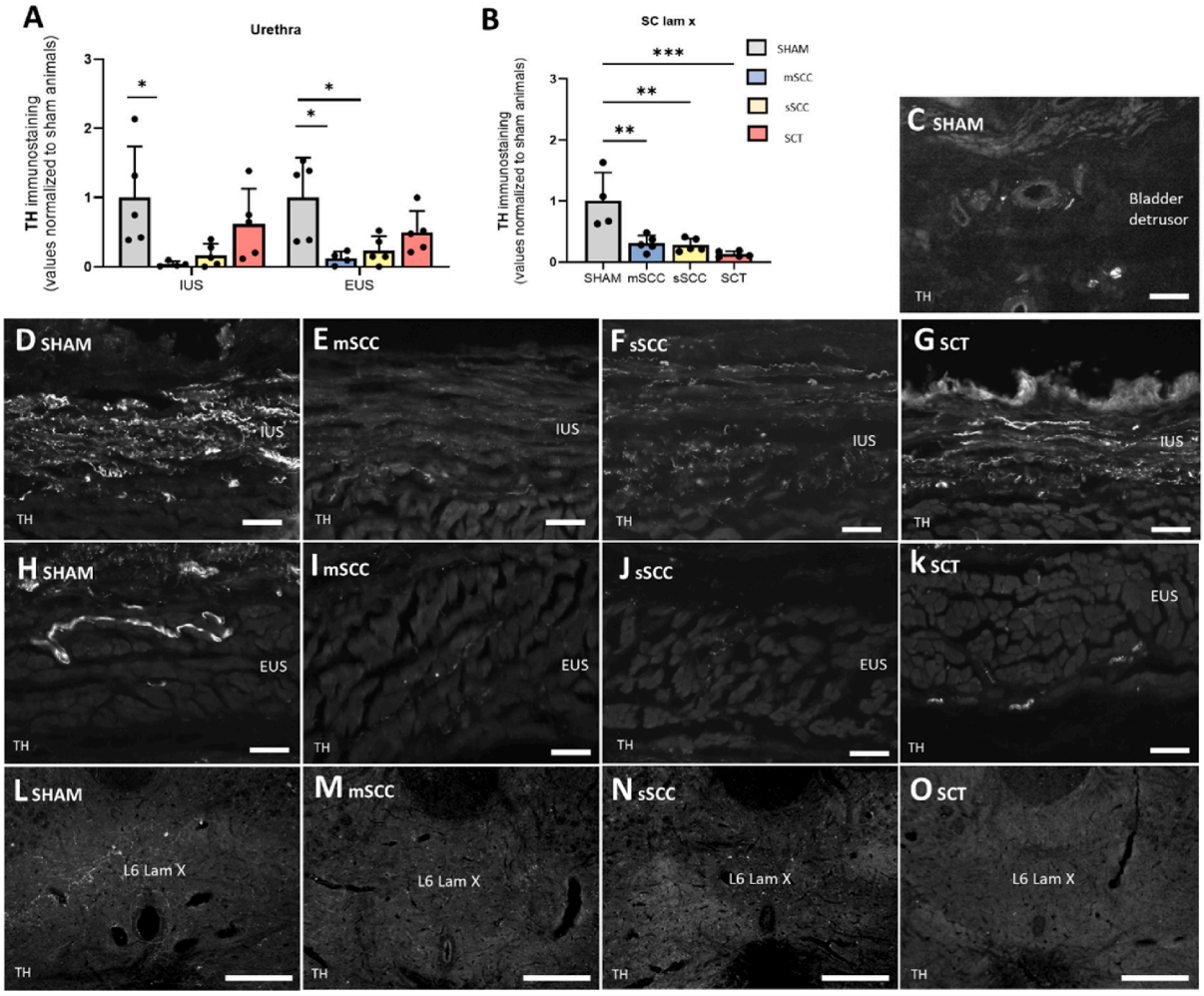

Fig. 7. Expression of Tyrosine Hydroxylase (TH) after mild and severe spinal cord contusion and spinal cord transection. Noradrenergic fibres were analysed by immunostaining against TH, a well-established marker of noradrenergic fibres. TH-immunoreactive fibres were investigated in the urethra (**A**) and L5-S1 spinal cord segments (**B**). In the bladder TH-positive profiles were scarce and only present in the vicinity of blood vessels (**C**). In the urethra, as no immunoreactive fibres were seen in the mucosa, analysis of the internal urethral sphincter (IUS) of SHAM (**D**), mSCC (**E**), sSCC (**F**) and SCT animals (**G**) showed a decrease in labelling only in mSCC animals. In the external urethral sphincter (EUS), assessed in SHAM (**H**), mSCC (**I**), sSCC (**J**) and SCT animals (**K**), loss of TH immunolabelling was found in sections from mSCC and SCC, but not in the SCT group. In the lumbosacral spinal cord, TH expression was present in the laminae X (Lam X) of SHAM (**L**), mSCC (**M**), sSCC (**N**) and SCT animals (**O**), showing a strong decrease of TH immunolabelling in SCI groups. Data were compared using One-Way ANOVA followed by Tukey's multiple comparison test (*$p < 0.01$, **$p < 0.01$, ***$p < 0.001$ versus SHAM). Scale bars equal 50 μm.

associated with improved LUT function (Breyer et al., 2017), which was not the case here. In turn, noradrenergic LUT innervation was studied by examining TH immunoreactivity, an enzyme involved in catecholamine biosynthesis. TH-positive profiles were very scarce in the bladder, with small fibres present in the vicinity of blood vessels. The paucity of TH fibres in the bladder was consistent with studies from other authors (Gosling et al., 1999; Persyn et al., 2016; Watanabe and Yamamoto, 1979). In the urethra, TH-positive fibres were present in the IUS and EUS and reduced after spinal trauma. The decrease in VAChT and TH expression in the LUT may reflect either a reduction in the synthesis of these proteins or injury of the nerve fibres, arising after SCI, even if this represents an indirect insult to bladder nerve fibres. Accordingly, a reduction in the expression of pan-neuronal markers has been observed in the bladder and urethra after spinal cord transection (Takahara et al., 2007).

Effect of different SCI models on the lumbosacral spinal cord

The development of SCI-induced bladder dysfunction is accompanied by neuroplasticity events at the lumbosacral spinal cord that lead to NDO development (Shimizu et al., 2023). Considering the different areas of tissue damage at the thoracic trauma site after contusion and transection, it was important to investigate its consequences on the lumbosacral cord, where important changes have been reported after SCT (Chambel et al., 2022). As in the LUT, axonal sprouting was investigated by assessing GAP43 levels. This protein was present in the superficial dorsal horn, where it increased after spinal trauma, in agreement with previous observations (Chambel et al., 2022; Frias et al., 2015). In lamina I-II, sprouting was more prominent after mSCC, likely indicating enhanced axonal sprouting. GAP43 expression was also upregulated in the IML, but without significant differences between the SCI groups, suggesting that IML sprouting was less dependent on the severity of spinal lesion. The superficial dorsal horn and the IML areas are known to receive sensory input originating in the bladder (Cruz et al., 2005; Cruz et al., 1994; Fuller-Jackson et al., 2021) and, though we did not perform co-localization analysis, it is likely at least part of GAP43-positive profiles are peptidergic sensory afferents, particularly in SCT animals (Frias et al., 2015), as GAP43 increase was accompanied by CGRP upregulation in the same spinal areas, more evident in sections from mSCC. This abnormal axonal expansion of peptidergic afferents has been shown to take place due to time- and NGF-dependent fluctuations in axon guidance cues and their receptors (Chambel et al., 2022).

Expression of VAChT was also investigated in the spinal cord. This marker is present in cholinergic cell bodies and terminals, and its expression can be used as a measure of neuronal injury (Maeda et al., 2004; Takahara et al., 2007). Indeed, we found a downregulation of VAChT expression in the IML, where pre-ganglionic neurons involved in micturition control are located (de Groat et al., 2015; Karnup, 2021). VAChT was also decreased in motor neurons located in the ventral horn, the Onuf's nucleus (Schellino et al., 2020), which are known to project and control the urethral sphincter. VAChT was reduced after SCI, particularly after SCT. This reduction correlated with urinary impairment, suggesting that bladder dysfunction also reflects changes in cholinergic cell bodies and fibres in the lumbosacral spinal cord. Moreover, the lack of supraspinal input induced by SCT led to the most marked decrease in VAChT expression, highlighting the important contribution of supraspinal centres to micturition control (Fowler et al., 2008).

Expression of TH at the lumbosacral spinal cord was also studied. The presence of this enzyme is considered a marker of descending noradrenergic fibres originating in the brainstem (Heinricher et al., 2009; Tavares et al., 2021; Westlund et al., 1983). TH-immunoreactive fibres were only seen in laminae X, in contrast with other studies in which TH-positive cells were observed in other areas of the spinal cord (Hou et al., 2016; Fuller-Jackson et al., 2021), possibly reflecting the use of different antibodies with distinct sensitivity. Nonetheless, TH levels were reduced in all SCI animals, particularly after complete transection. This reflects a lack of supraspinal input to the lumbosacral spinal cord, irrespective of the area of damaged tissue.

Like all studies, there are also limitations that should be addressed. While the majority of SCI patients are male (Chen et al., 2016), female rats were used, as in most experimental studies. The reason for this resides in anatomical differences between sexes, with males presenting prostate and longer urethras. Because SCI is followed by a period of little or no bladder reflex activity (Anderson et al., 2023; Bywater et al., 2018), animals need to be submitted to abdominal compression for urine removal until automatic micturition develops. Abdominal compression is more easily done in female than male rats, which presents an increased risk of obstructions, urinary infection and, eventually, kidney failure. We acknowledge that in future studies male animals should be considered for a proper representation of human SCI. Moreover, one should also take into consideration that traumatic lesions of the spinal cord (compression, contusion, transection, hemisection) constitute the most widely used animal models of SCI but spinal lesions can also arise from degenerative age-related disorders, including degenerative joint disease. Therefore, future experimental and translational studies should address these non-traumatic SCIs as they are under investigated and require additional scientific investment.

Conclusions

Human spinal cord injuries vary between patients and produce different outcomes, which is widely acknowledged amongst clinicians and researchers. Most SCIs are accompanied by urinary impairment, resulting from NDO and DSD. Experimental SCI models have classically used spinal transections to clarify pathophysiological mechanisms and devise new therapeutic strategies, despite spinal contusions being more frequent. It is, thus, important, to compare changes in LUT function and peripheral and central neuroplasticity following spinal transection and contusion. For several years, this issue has been investigated (Breyer et al., 2017; Mitsui et al., 2014; Pikov et al., 1998), and here, we built on earlier findings and expand observations to the urethra and lumbosacral spinal cord, a major hub for post-SCI neuroplasticity. To our knowledge, this is the first study comparing the effects of different models of SCI at both spinal cord and LUT levels that include the analysis of urethral tissue.

We devised an affordable setup that produces reproducible contusions and have validated its use. We compared LUT function and tissue integrity at the lesion site and found a correlation between injury severity and bladder dysfunction and the area of damaged tissue. At the LUT, mild and severe contusions and transection produced similar changes in innervation, whereas at the lumbosacral dorsal horn less severe injury was accompanied by axonal sprouting, particularly of peptidergic afferents, eventually linked to changes in sensory function. This data shows that changes in LUT innervation and dysfunction arising after spinal cord injury by contusion and transection are similar but result from different neuroplastic events at the lumbosacral spinal cord. This could have implications for the future development of therapeutic tools for urinary impairment after SCI.

CRediT authorship contribution statement

Ana Ferreira: Writing – review & editing, Writing – original draft, Visualization, Methodology, Formal analysis, Conceptualization. **Sílvia Sousa Chambel:** . **António Avelino:** Methodology. **Diogo Nascimento:** Methodology. **Nuno Silva:** Methodology. **Célia Duarte Cruz:** .

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Publication III

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Article

Spinal Cord Injury Causes Marked Tissue Rearrangement in the Urethra—Experimental Study in the Rat

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Abstract: Traumatic spinal cord injury (SCI) results in the time-dependent development of urinary impairment due to neurogenic detrusor overactivity (NDO) and detrusor-sphincter-dyssynergia (DSD). This is known to be accompanied by massive changes in the bladder wall. It is presently less clear if the urethra wall also undergoes remodelling. To investigate this issue, female rats were submitted to complete spinal transection at the T8/T9 level and left to recover for 1 week and 4 weeks. To confirm the presence of SCI-induced NDO, bladder function was assessed by cystometry under urethane anesthesia before euthanasia. Spinal intact animals were used as controls. Urethras were collected and processed for further analysis. Following thoracic SCI, time-dependent changes in the urethra wall were observed. Histological assessment revealed marked urethral epithelium reorganization in response to SCI, as evidenced by an increase in epithelial thickness. At the muscular layer, SCI resulted in strong atrophy of the smooth muscle present in the urethral sphincter. Innervation was also affected, as evidenced by a pronounced decrease in the expression of markers of general innervation, particularly those present in sensory and sympathetic nerve fibres. The present data show an evident impact of SCI on the urethra, with significant histological rearrangement, accompanied by sensory and sympathetic denervation. It is likely that these changes will affect urethral function and contribute to SCI-induced urinary dysfunction, and they deserve further investigation.

Keywords: epithelium; nerve fibres; micturition; spinal cord injury; urethra; urethral sphincter

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1. Introduction

Micturition, defined as the coordinated process of urine storage by the urinary bladder and its periodic elimination by the urethra, is mediated by complex neuronal mechanisms involving central and peripheral neurons. The complexity of the neuronal mechanisms controlling lower urinary tract (LUT) function renders them vulnerable to injuries affecting the central nervous system, such as spinal cord injuries (SCIs) [1,2]. Traumatic SCIs are typically followed by a period of little or no bladder activity during the acute phase of the disease [3,4], resulting from the interruption of ascending and descending circuits responsible for regulating LUT function. After injury, several mechanisms are set in motion, and cells are recruited to promote the sealing and healing of the injury site [5,6]. With time, neuroplastic mechanisms operating at the lumbosacral spinal cord lead to the emergence of an alternative reflex pathway responsible for NDO [7,8]. NDO is characterized by strong and frequent involuntary bladder contractions and often courses with detrusor-sphincter-dyssynergia (DSD: a lack of coordination between the bladder and urethral sphincter contraction, causing urinary incontinence episodes) [1]. Despite the reduction in mortality and SCI-associated morbidities, reflecting the development of better pharmacological and surgical interventions, there is no efficient treatment to fully overcome

SCI-induced incontinence symptoms. Available treatments aim to protect kidney function, reduce urinary tract infections, and promote continence. Typically, antimuscarinic drugs are initiated to reduce bladder overactivity and intravesical pressures [9,10], and this is often combined with intermittent self-catheterization. For refractory cases, intradetrusor injections of botulinum toxin remain the gold standard therapy [11,12]. Nonetheless, all these therapies are accompanied by bothersome side effects and may lose efficiency over time, leading to treatment discontinuation. Therefore, a breakthrough is urgently needed. New therapeutic strategies are currently being developed, some without a direct focus on the LUT [13,14], and others are being used to control bladder function in spinal intact patients with LUT dysfunction [15], but the effects on SCI-induced urinary impairment have yet to be investigated.

The development of NDO and DSD is accompanied by considerable post-SCI tissue reorganization in the bladder wall. It is known that detrusor muscle cells become hypertrophic, with thick bundles of connective tissue coursing between muscle fibers [16,17]. Changes in bladder innervation have also been described, including the impairment of parasympathetic fibres and the upregulation of sensory innervation following SCI [16,18]. In contrast, the consequences of SCI in tissue organization within the urethral wall have been less well addressed. The urethra is a tubular organ, traditionally seen as the mere outlet for urine removal. In the proximal urethra, i.e., closer to the urinary bladder, the mucosa is lined with an epithelium with transitional properties, gradually changing to a stratified lining epithelium towards the distal urethra. Beneath the lamina propria, the urethral sphincter consists of two muscular layers. The inner layer, the internal urethral sphincter (IUS), which is composed of smooth muscle fibres extending from the bladder, is under the control of the autonomic nervous system. The outer muscular layer, the external urethral sphincter (EUS), controlled by the somatic nervous system, is composed of striated muscle cells and is responsible for voluntary sphincter closure during storage.

Accumulating evidence indicates that the urethra plays a role in LUT function rather than simply serving as a conduit for urine expulsion. In humans and animal models, different protocols of electrical stimulation of the urethra alter bladder activity [19,20]. In awake ewes and humans, urine flow along the urethra facilities bladder contractions, while urethral anesthesia impairs bladder emptying [21,22]. More recently, the presence of specialized epithelial cells, which release peripheral neurotransmitters involved in urethra–vesical crosstalk, has also been demonstrated [23,24]. Although undescribed, it is likely that the urethra might be affected by traumatic SCI, contributing to the impairment of LUT function. Thus, the present study sought to investigate time-dependent changes in the tissue organization of the urethral wall and in nerve fibers coursing in the mucosa and sphincter.

2. Results

2.1. Complete Spinal Cord Transection Alters Bladder Reflex Activity

Before tissue collection, all animals were submitted to cystometries under urethane anesthesia. Spinal intact (INT) animals presented normal bladder function (Figure 1A,D–F; Table 1). One week after the spinal lesion, bladder reflex contractions were almost abolished (Figure 1B,D–F; Table 1). At 4 weeks post-SCI, it was possible to observe frequent and high-amplitude bladder reflex contractions, with a typical pattern of detrusor hyperactivity (Figure 1C–F; Table 1).

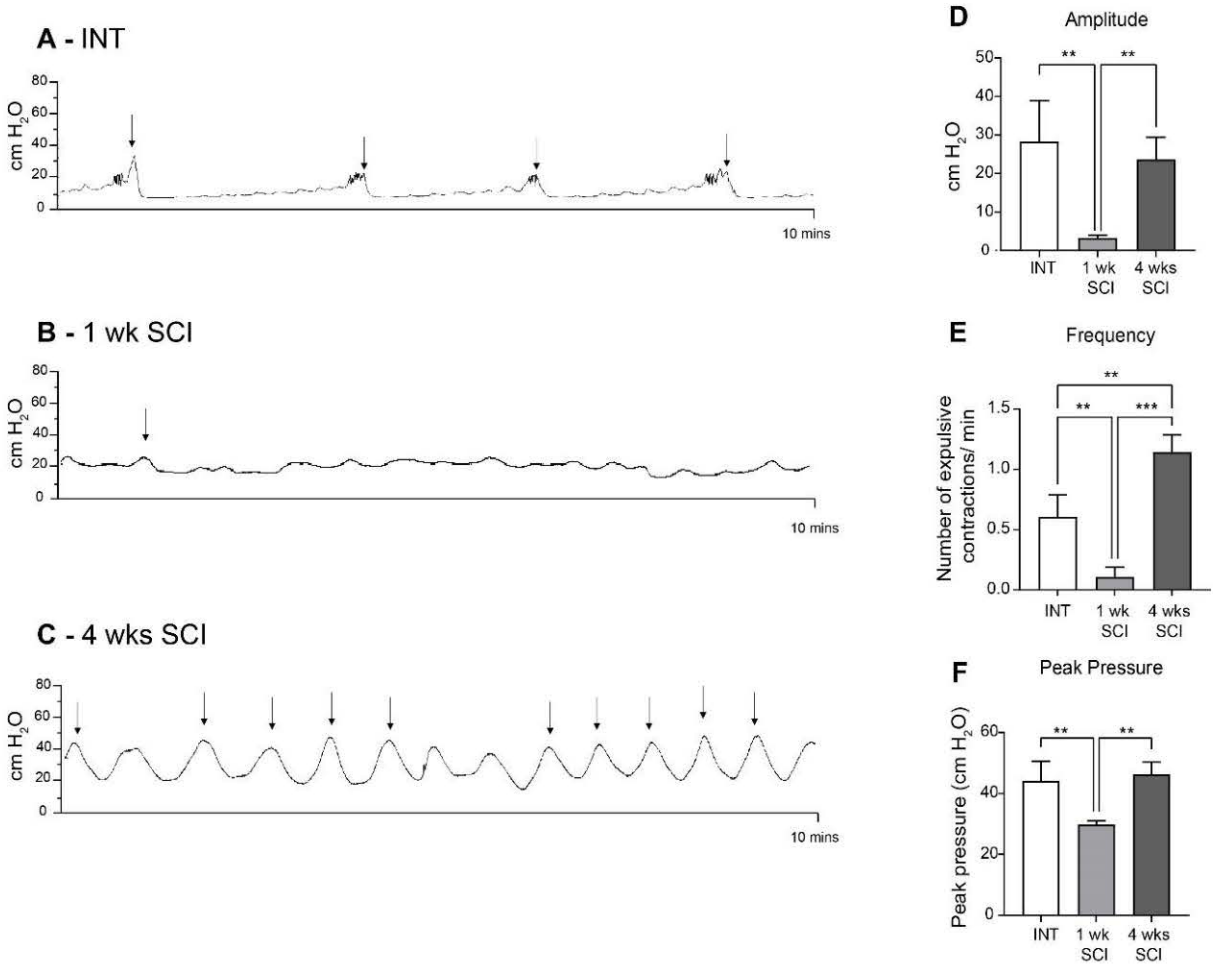


Figure 1. Bladder reflex activity. Bladder contractility was assessed by cystometry under urethane anesthesia. Intact animals (**A**) presented a typical bladder function. One week post-SCI (**B**), SCI animals were considered to be in spinal shock, with an almost total absence of bladder contractions. In 4-week SCI rats (**C**), bladder contractions were partially restored, with a pattern of bladder hyperreflexia. Urodynamic parameters, including the amplitude (**D**), frequency (**E**), and peak pressure (**F**) of the expulsion bladder contractions of the overall experimental groups ($n = 4$). One-way ANOVA followed by Tukey's multiple comparison test (** $p < 0.01$; *** $p < 0.001$;). The arrows indicate the expulsion of a drop of urine.

Table 1. Urodynamic parameters from intact and SCI rats. Cystometric values of frequency, peak pressure, and amplitude of voiding contractions of spinal intact and SCI animals (1 and 4 weeks post-SCI). Cystometrograms were analyzed using the LabScribe software. Only contractions that reached at least 10 cm H_2O of amplitude were considered for quantification. Data are represented as the mean ± standard deviation (one-way ANOVA followed by Turkey's multiple comparison test; ** $p < 0.01$ compared to intact; #### $p < 0.0001$, ## $p < 0.01$ compared to 1w SCI).

	Spinal Intact	SCI—1 Week	SCI—4 Weeks
Frequency	0.60 ± 0.18	0.10 ± 0.08 ** $p < 0.01$	1.14 ± 0.15 ** $p < 0.01$ #### $p < 0.0001$
Peak pressure	44.27 ± 6.34	29.77 ± 1.29 ** $p < 0.01$	46.27 ± 4.06 ## $p < 0.01$
Amplitude	28.31 ± 10.63	3.29 ± 0.68 ** $p < 0.01$	23.65 ± 5.62 ## $p < 0.01$

2.2. Spinal Cord Transection Affects the Organization of the Urethral Epithelium

To assess the consequences of SCI in the tissue organization of the urethral wall, longitudinal urethral sections were stained with haematoxylin and eosin. Measurements of the thickness of each histological layer were performed where indicated (Figure 2A). Urethral sections from spinal intact animals showed evidence of a stratified epithelium, with several layers, the most superficial of which was composed of flattened cells (Figure 2B). The epithelium thickness was 0.96 ± 0.47 µm (Figure 2E). One week after spinal injury, epithelial disorganization was evident, with signs of cellular loss in the most superficial layers (Figure 2C). The thickness of the epithelium was 1.3 ± 0.32 µm (Figure 2E). Four weeks after spinal lesion, epithelial layers were again evident, with fewer signs of cellular loss and a higher number of superficial flattened cells (Figure 2D). The epithelial height was 1.80 ± 0.34 µm (Figure 2E; $p < 0.05$ vs. INT). The thickness of the lamina propria remained unaltered (Figure 2F).

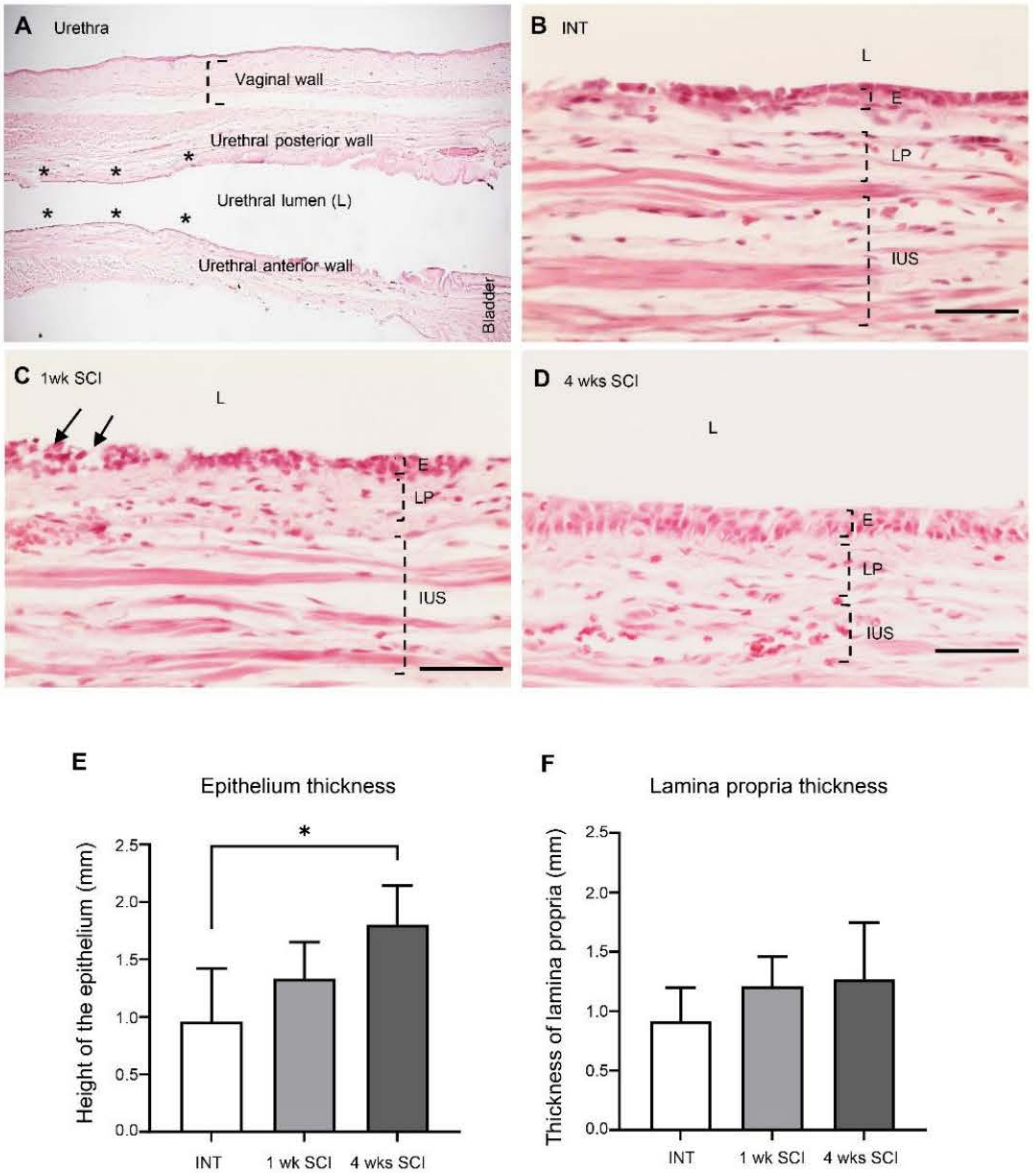


Figure 2. Epithelium and lamina propria organization. The morphology of the urethral wall was studied using hematoxylin and eosin-stained urethral sections. A general view of the urethra is represented in (**A**). The localizations of the vaginal and the urethral wall (posterior and anterior), the urethral lumen, and the bladder are identified. The six locals chosen for measures are marked as *.

Intact animals (**B**) presented a well-organized epithelium (**E**) and lamina propria (LP). One-week (1 wk SCI) spinal injured rats (**C**) presented severe damage in the mucosa, with some signs of cell desquamation (pointed by arrows) in the E and some bleeding of the LP (not shown). Four weeks (4 wks SCI) after the spinal insult (**D**), the E and LP recovered its organization. Scale bars equal 50 µm. Quantification by Image J of the height of the epithelium (**E**), showing a significant increase in 4-weeks SCI animals. No changes were found in the thickness of the lamina propria (**F**) (n = 4). One-way ANOVA followed by Tukey's comparison test (* $p < 0.05$).

2.3. Changes in the Urethral Sphincter after SCI

2.3.1. Internal Urethral Sphincter

Paraffin-embedded urethral sections were stained with Sirius Red to investigate the collagen content in the urethral wall (Figure 3A–C,G). In the IUS, Sirius red staining showed no differences in stained connective tissue, with bundles of connective fibers, most likely collagen, coursing between smooth muscle fibers (Figure 3A–C,G).

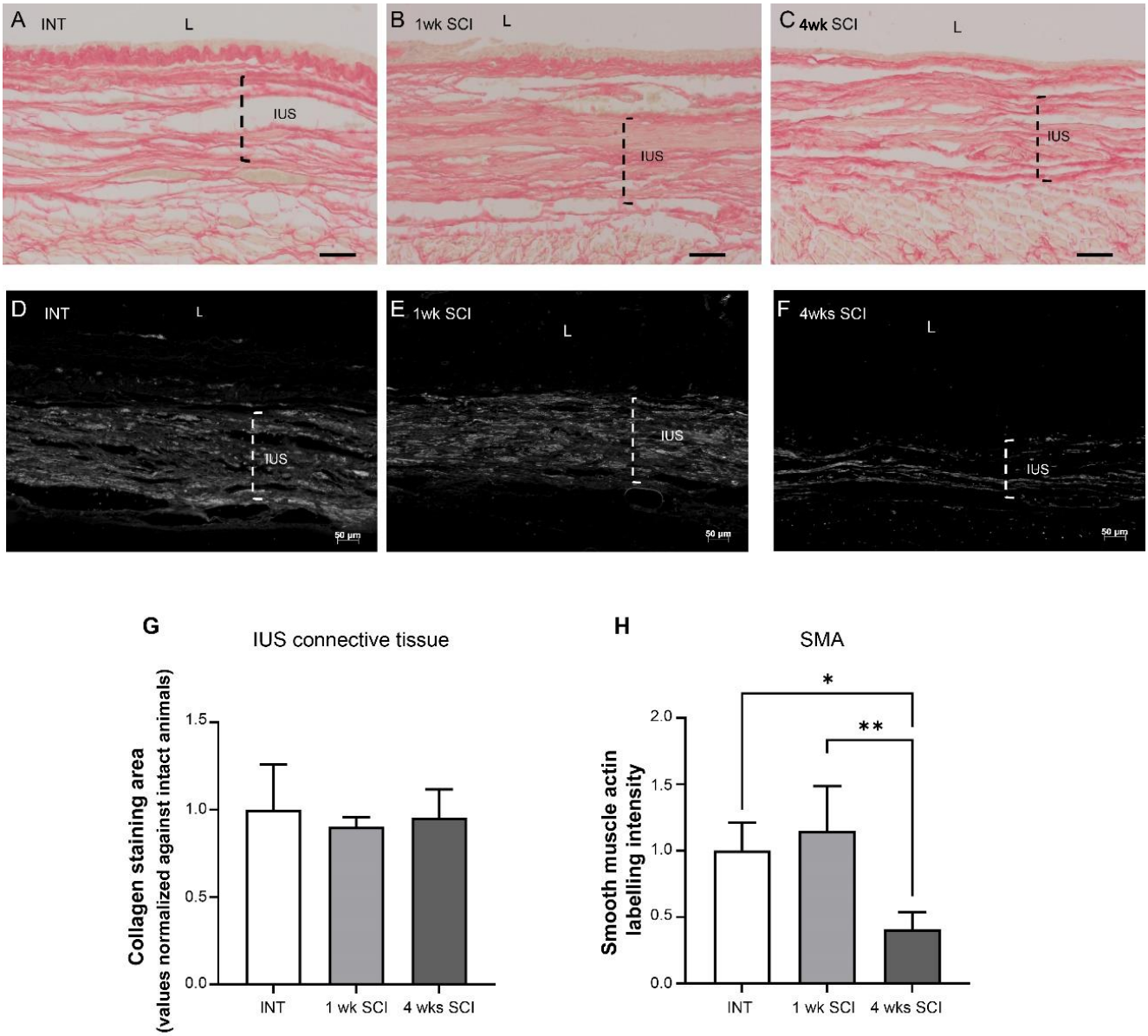


Figure 3. Internal urethral sphincter integrity. The integrity of the internal urethral sphincter (IUS) was measured by Sirius Red staining. Intact (**A**), 1 wk SCI (**B**) and 4 wks SCI (**C**) animals presented no

changes in connective tissue arrangement (**G**). Immunostaining of smooth-muscle actin (SMA) showed that spinal intact (**D**) and 1-week SCI animals (**E**) presented a tick IUS with strong actin expression. In sections from animals left to recover 4 weeks after spinal lesion (**F**), there was evidence of a marked decrease in SMA expression. The quantification of the IUS labelling intensity of actin in the proximal urethra (**H**) ($n = 4$). One-way ANOVA followed by a Tukey's multiple comparison test (* $p < 0.05$ versus INT; ** $p < 0.01$ versus 1-week SCI).

The expression of smooth muscle actin (SMA) was also analyzed in paraffin-embedded urethral sections. The abundant expression of SMA was found in INT animals (Figure 3D,H), and similar observations were made in sections obtained from 1-week post-SCI animals (Figure 3E,H). In contrast, SMA expression was significantly reduced in sections from 4-weeks post-SCI animals (Figure 3F,H; $p < 0.05$ vs. INT and $p < 0.01$ vs. 1 week post-SCI).

2.3.2. External Urethral Sphincter

The effects of SCI on the arrangement of the external urethral sphincter were also analysed. In INT sections stained with haematoxylin–eosin, it was possible to observe long striated muscle fibers (Figure 4A). The average transverse area of striated fibres was 10.48 ± 4.8 μm^2 (Figure 4G). Observations were also made in sections from 1-week and 4-weeks post-SCI animals (Figure 4B,C), and the average transverse areas, respectively, were 8.52 ± 1.00 and 6.43 ± 0.73 μm^2 (Figure 4G), suggesting a trend towards slimmer muscle fibres that did not reach statistical significance. Sirius red staining showed significant signs of fibrosis in SCI animals, with a thick bundle of connective fibres between striated muscle cells evident at 1 and 4 weeks post-SCI (Figure 4D–F,H; * $p < 0.05$ and ** $p < 0.01$ versus INT).

2.4. Urethral Innervation after SCI

2.4.1. General Innervation

Changes in urethral innervation associated with SCI were investigated by immunostaining urethral sections from INT and SCI rats. General innervation was evaluated by the expression of β-III tubulin, a pan-neuronal marker (Figure 5A–C). In the mucosa, no significant differences in β-III tubulin expression were found (Figure 5A–C,A1–C1,D). In contrast, a reduction was found in the urethral sphincter. In the IUS, while β-III tubulin remained unaltered at 1 week post-SCI (Figure 5B,B2,E) in comparison with INT (Figure 5A,A2,E), a significant decrease was observed 4 weeks following the spinal lesion when compared to INT and 1-week post-SCI animals (Figure 5C,C2,E; $p < 0.05$ vs. INT and 1w SCI). In the EUS, values did not reach statistical significance, although a tendency for early denervation was already observable at 1 week following the spinal lesion (Figure 5B,B3,C,C3,F).

2.4.2. Sensory Innervation

As β-III tubulin levels suggested the occurrence of the denervation of the urethral wall, the effects of SCI on the sensory fibres present were assessed by evaluating calcitonin gene-related peptide (CGRP) immunostaining. In sections from spinal intact animals, CGRP-positive fibres were present predominantly in the mucosa, with fibres penetrating the epithelial layer and coursing in the lamina propria (Figure 6A,A1). After SCI, CGRP expression in the mucosa was significantly decreased at both time points after the spinal lesion (Figure 6B,B1,C,C1,D; $p < 0.0001$ vs. INT for both time points). In the muscular layer of INT animals, CGRP expression was less abundant (Figure 6A). As in the mucosa, CGRP levels in the IUS were also decreased after SCI (Figure 6B,C,E $p < 0.01$ vs. INT for both time points). This reduction was also evident in the EUS (Figure 5B2,B3,F; $p < 0.01$ vs. INT for both time points).

2.4.3. Sympathetic Innervation

Sympathetic innervation was evaluated by tyrosine hydroxylase (TH) immunostaining. In intact animals, no TH-positive fibres were found in the mucosa (Figure 7A), and

immunoreactive fibres were predominantly distributed in the IUS (Figure 7A1). Some positive profiles were also present in the EUS (Figure 7A2). One week and four weeks after the spinal lesion, TH immunoreactive fibres in the IUS were significantly reduced (Figure 7B1,C1,D; $p < 0.05$ vs. INT). In the EUS, no differences were found between the experimental groups (Figure 7A2–C2,E).

2.4.4. Parasympathetic Innervation

Parasympathetic innervation in the urethra was assessed by immunostaining against VAChT. In intact animals, parasympathetic tracts were observed mostly at the mucosa (Figure 8A,A1) and in the IUS (Figure 8A,A2), with no signs of VAChT-positive fibres in the EUS. After SCI, no significant changes were observed in the mucosa (Figure 8D). In the IUS, there was a trend for an increased intensity of VAChT immunostaining that did not reach statistical significance (Figure 8E), with abundant positive profiles observed in the mucosa and in the IUS at 1 (Figure 8B,B1,B2) and 4 weeks post-SCI (Figure 8C,C1,C2).

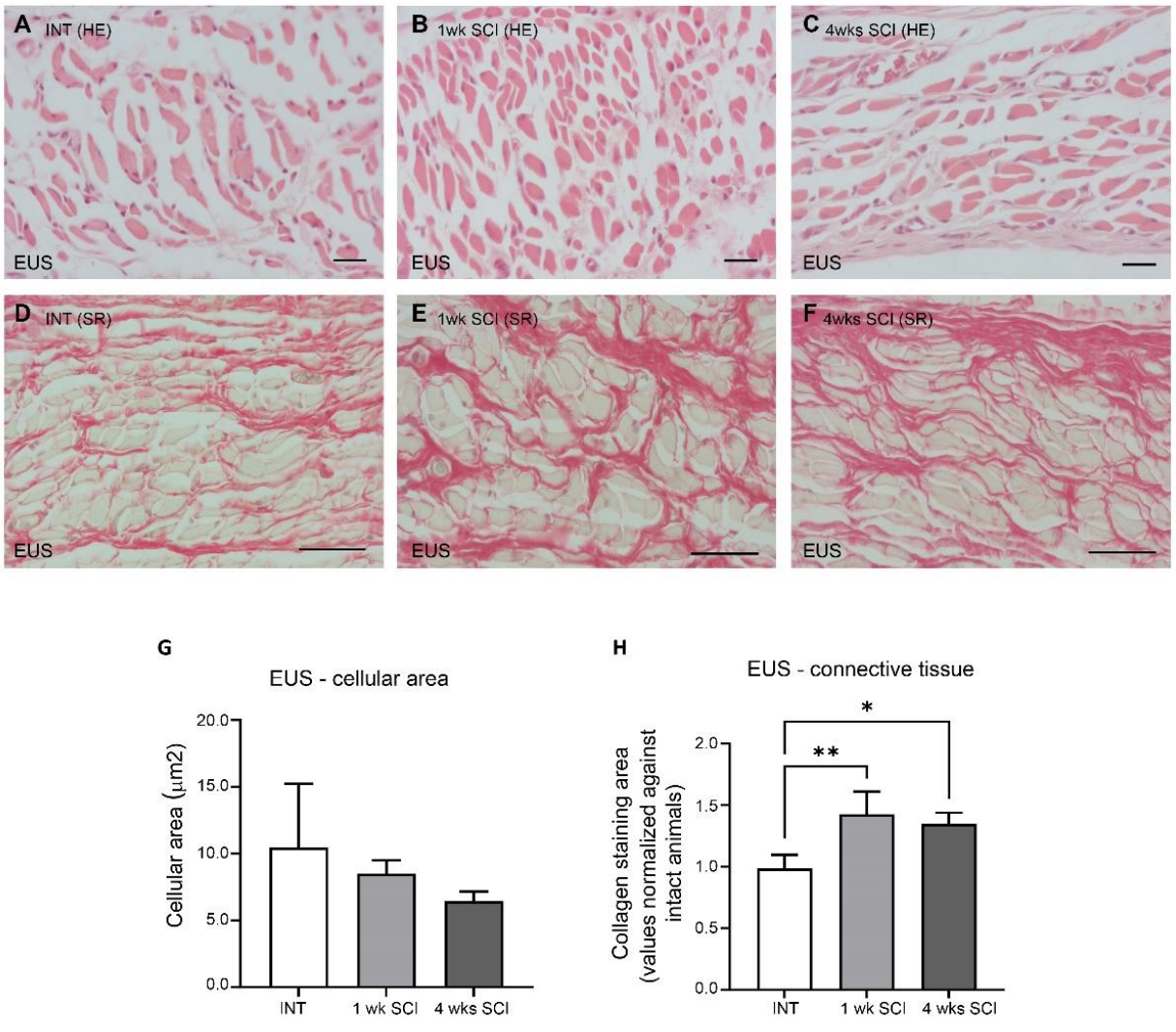


Figure 4. External urethral sphincter integrity. The morphology of the external urethral sphincter (EUS) striated cells was assessed by staining paraffin-embedded sections with haematoxylin–eosin. Spinal intact animals (**A**) presented long and slender striated cells, as well as 1-week (**B**) and 4-weeks SCI animals (**C**), with no changes in the cellular area (**G**). The levels of fibrosis, estimated by the collagen content stained with Sirius Red, significantly increased in SCI animals at 1 week post-SCI (**E**) and were still evident at 4 weeks after spinal lesion (**F**) when compared to controls (**D**,**H**) (* $p < 0.05$; ** $p < 0.01$). Scale bars equal 50 µm. One-way ANOVA followed by a Tukey's multiple comparison test.

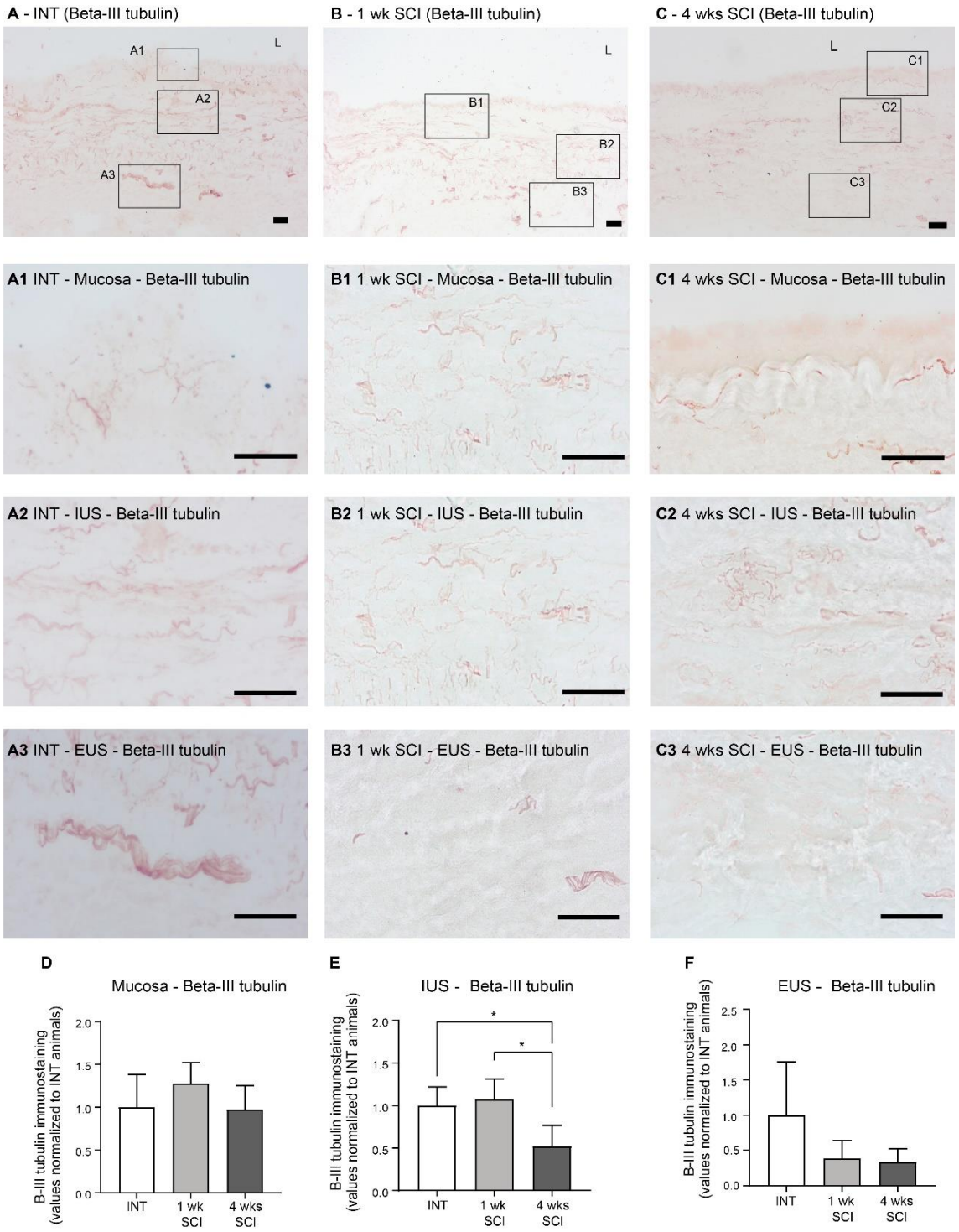


Figure 5. Immunolabeling of β-III tubulin in the proximal urethra. The general neuronal content was assessed using the ABC method for the detection of β-III tubulin, which stained the overall population of neuronal fibers in the urethral wall. Spinal intact animals (**A**) presented fibers widely distributed along the wall, in the mucosa (**A1**), in the internal urethral sphincter (IUS) (**A2**), and in the external urethral sphincter (EUS; (**A3**)). One-week SCI animals (**B**) presented an evident denervation in the EUS (**B3**) but not in the mucosa (**B1**) and IUS (**B2**). Animal lefts to recover 4-weeks post-SCI (**C**) presented denervation both in IUS (**C2**) and EUS (**C3**) but not in the mucosa (**C1**). Scale bars equal 50 µm. The quantification of labeling intensity by Image J in the mucosa (**D**), IUS (**E**), and EUS (**F**) (n = 5). One-way ANOVA followed by Tukey's multiple comparison test (* $p < 0.05$ versus spinal intact animals).

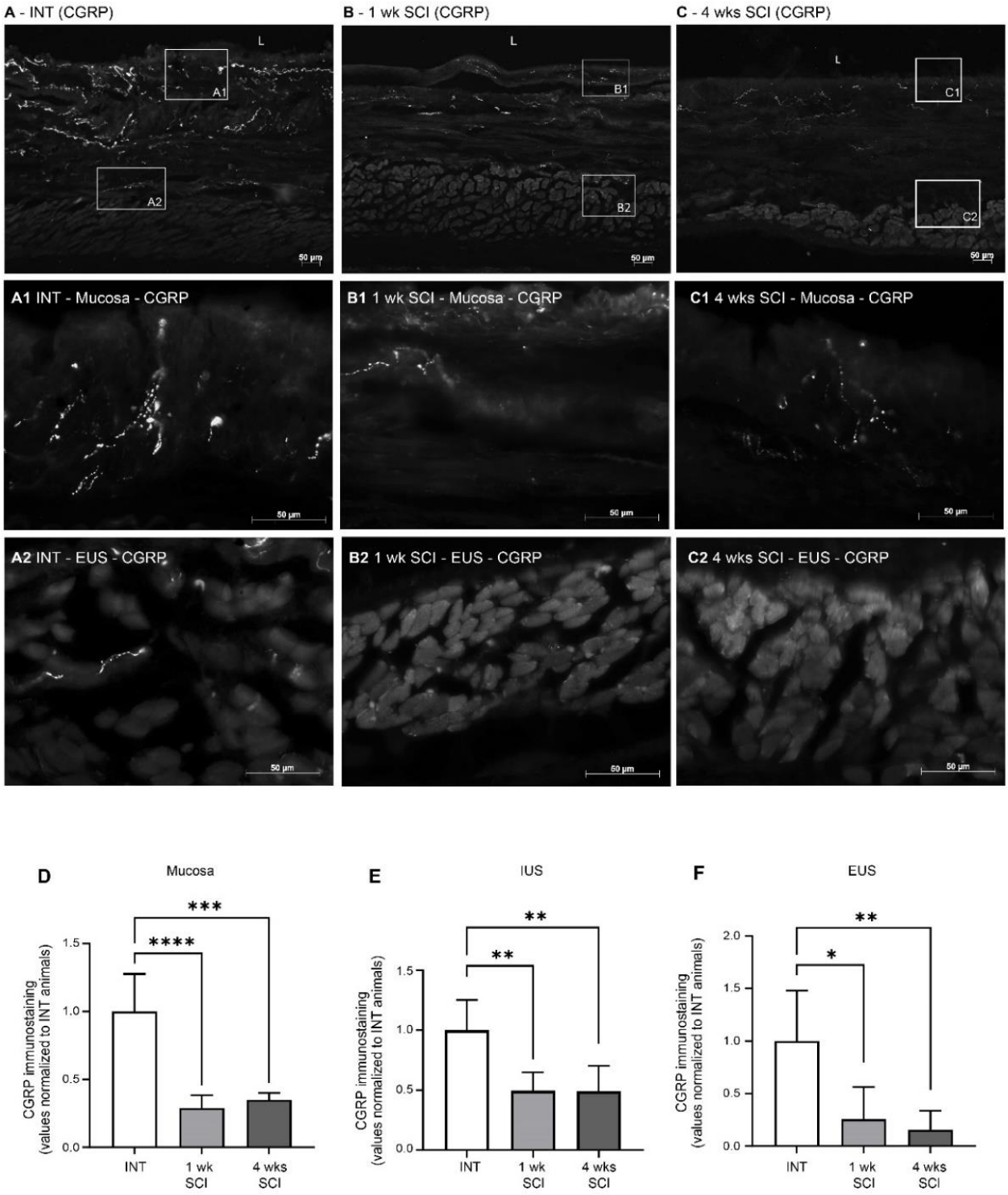
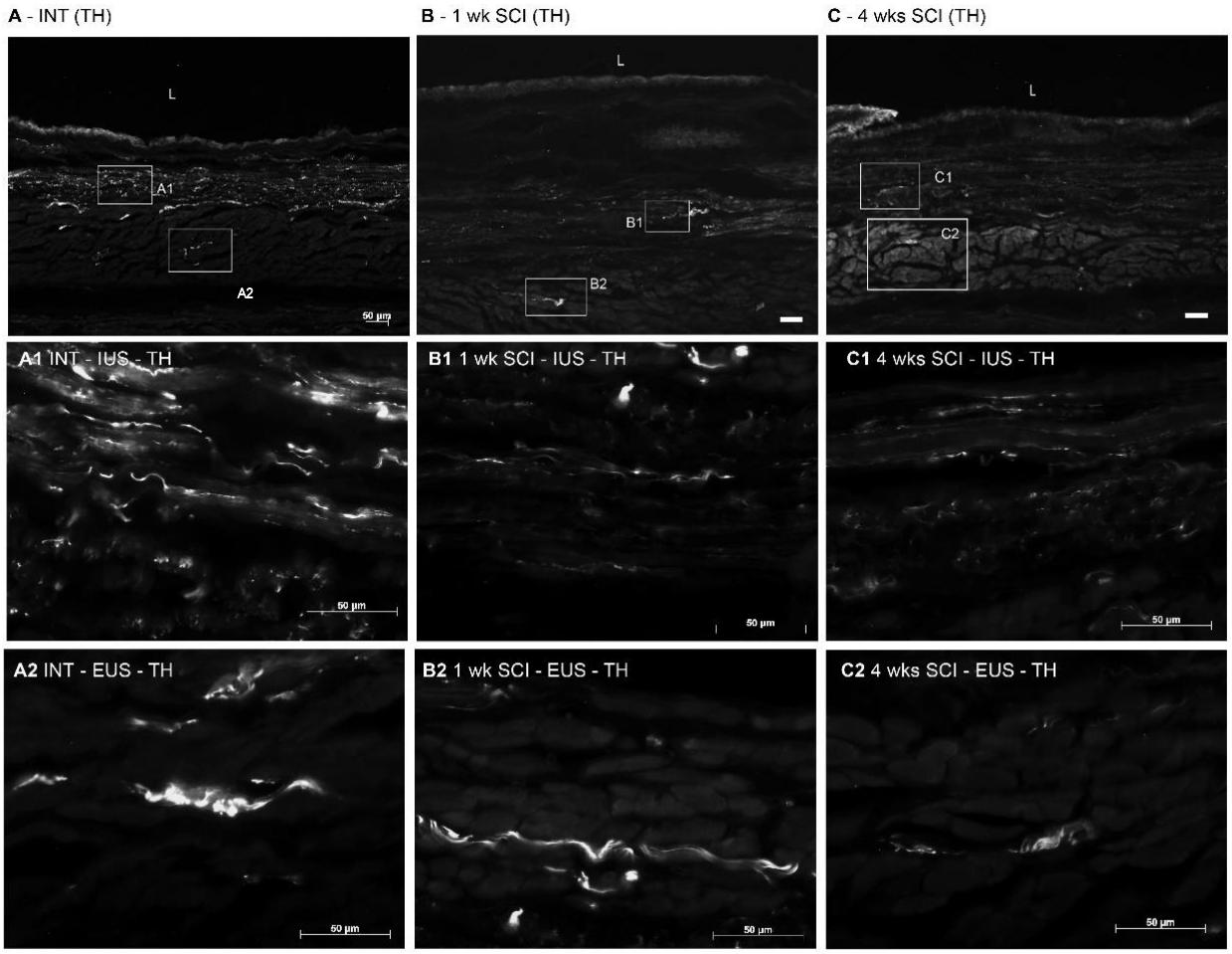


Figure 6. Distribution of CGRP-positive fibres in the proximal urethra. Sensory innervation was assessed by the immunostaining of the CGRP (calcitonin gene-related peptide) present in sensory peptidergic fibres. Intact animals (**A**) presented peptidergic fibres in the mucosa (**A1**), and some penetrated the epithelium (**E**) and through the lamina propria (LP). Some CGRP-positive fibres were also present in the external urethral sphincter (EUS) (**A2**). One- and four-weeks SCI animals ((**B**,**C**), respectively) presented a marked decrease in CGRP expression in the mucosa (**B1**,**C1**) and in the EUS area (**B2**,**C2**). Scale bars equal 50 µm. Quantification of labelling intensity by Image J in the mucosa (**D**), IUS (**E**), and EUS ((**F**) $n = 5$). One-way ANOVA followed by Tukey's multiple comparison test (**** $p < 0.001$; *** $p < 0.0001$, ** $p < 0.01$; * $p < 0.05$ versus spinal intact animals).



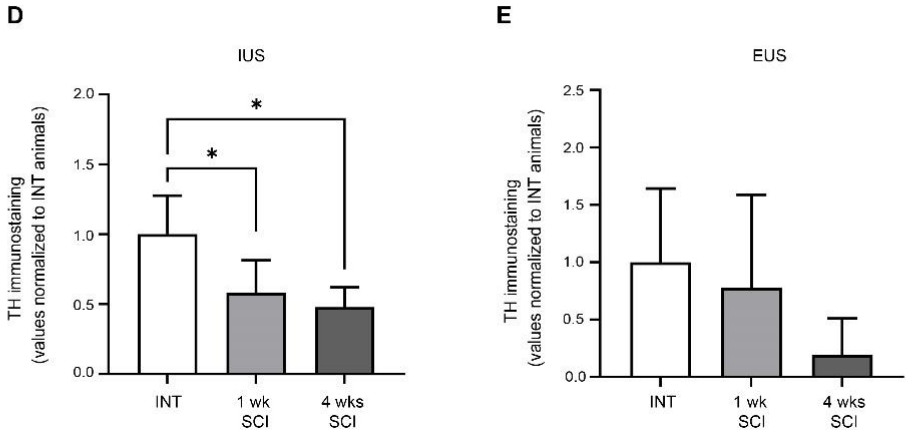
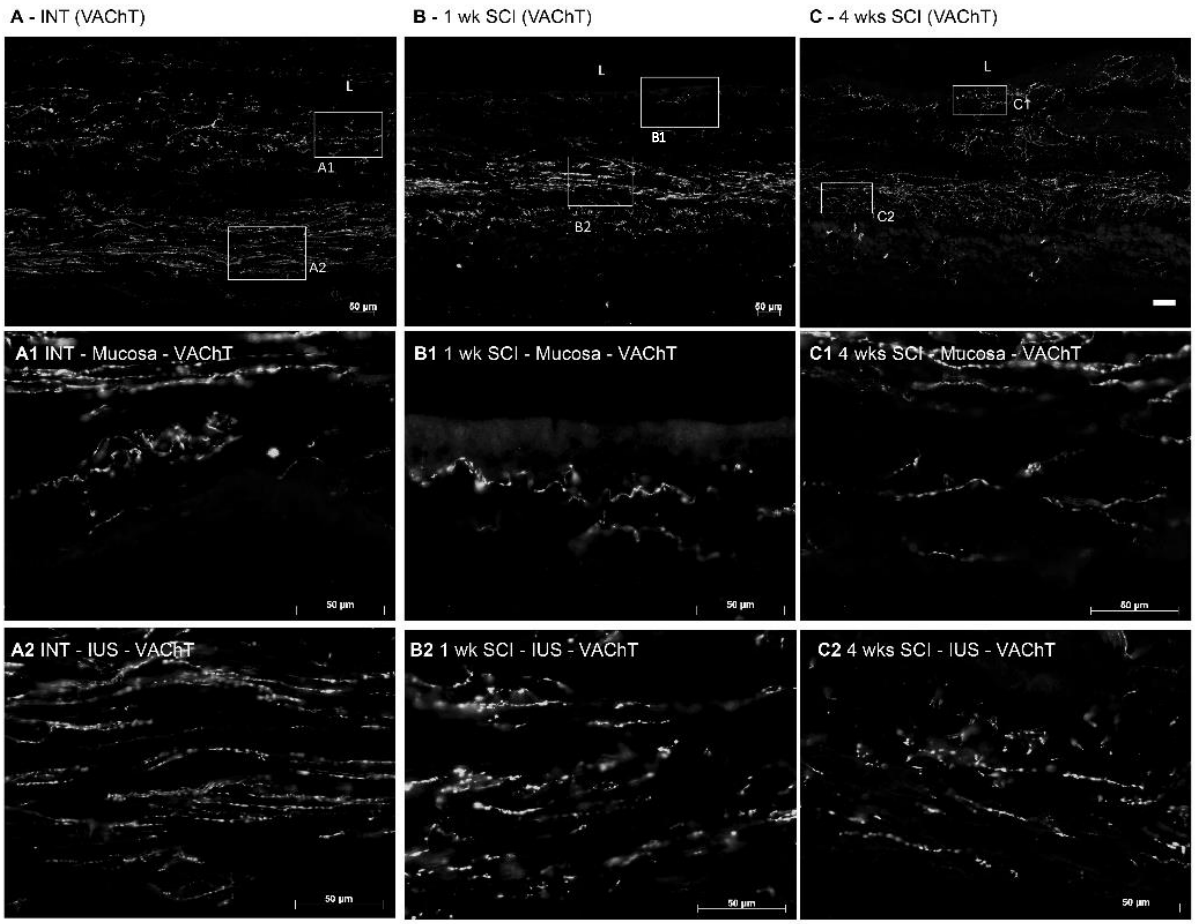


Figure 7. Expression of tyrosine hydroxylase (TH) in the urethral sphincter. Sympathetic innervation was analysed using TH immunohistochemistry, a noradrenergic marker for sympathetic fibres. Intact animals (**A**) presented TH-positive fibres predominantly in the internal and external urethral sphincter (IUS, (**A1**); EUS, (**A2**), respectively). No TH fibres were observed in the mucosa. Positive fibres were also present in sections from 1-week SCI animals (**B**,**B1**,**B2**), in which a prominent reduction was observed. This sympathetic denervation was also evident in sections from 4-weeks post-SCI animals (**C**), both in the IUS (**C1**) and EUS (**C2**). Scale bars equal 50 µm. The quantification of labelling intensity by Image J in the IUS (**D**) and EUS (**E**) (n = 4). One-way ANOVA followed by Tukey's multiple comparison test (* $p < 0.05$ vs. INT).



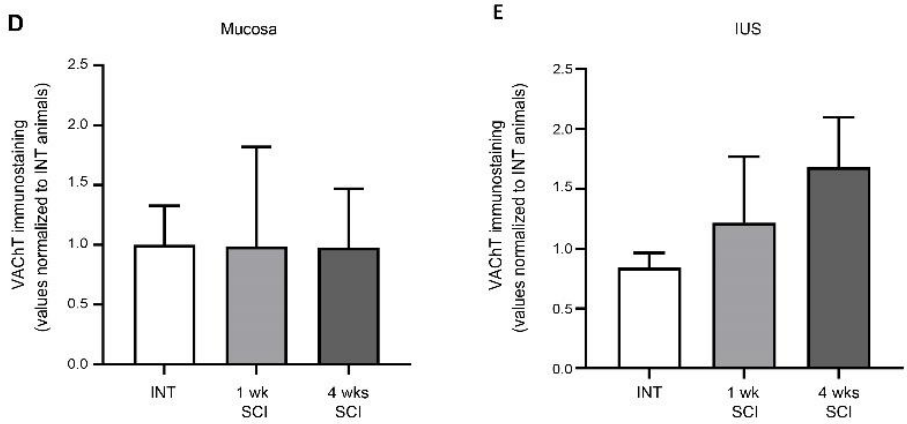


Figure 8. Immunolabeling of VAChT in the proximal urethra. Parasympathetic innervation was assessed by immunohistochemistry against the Vesicular Acetylcholine Transporter (VAChT), which stains cholinergic fibres. Intact animals (**A**) presented sympathetic fibres in the mucosa, penetrating the epithelial layer (**E**) and expanding in the lamina propria (LP) (**A1**). Immunoreactive fibres were also present in the internal urethral sphincter (IUS) (**A2**). A similar pattern was found in 1-week post-SCI animals (**B**,**B1**,**B2**). In sections from 4-weeks post-SCI animals, there was a trend towards an increase in VAChT immunoreactivity (**C**) in the mucosa and internal urethral sphincters ((**C1**); IUS, (**C2**), respectively). No VAChT fibres were observed in the EUS. Scale bars equal 50 µm. No significant changes were found in the mucosa (**D**) or IUS (**E**). The quantification of labelling intensity by Image J in the extension of the proximal urethra (n = 4). One-way ANOVA followed by Tukey's multiple comparison test.

2.4.5. Sprouting of Nerve Fibers

As axonal sprouting in the bladder accompanies NDO emergence [25,26], GAP-43 expression in the urethra was assessed. We found no significant differences between the experimental groups (Figure 9A–C,I) and observed the co-localization of GAP-43 with all markers of nerve fibres (Figure 9D–H), irrespective of the time point of disease progression.

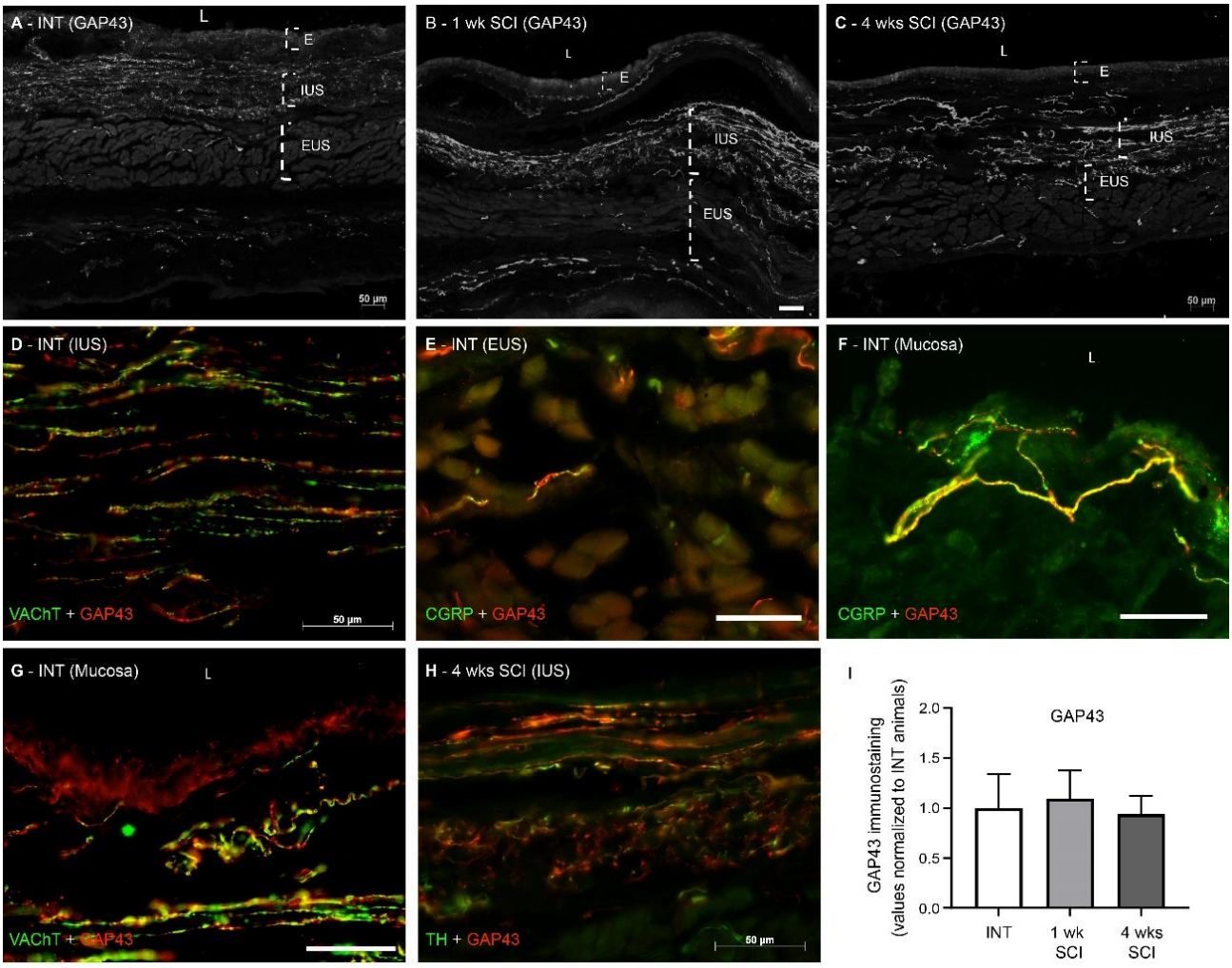


Figure 9. Immunolabeling of GAP-43 in the proximal urethra. The existence of neuronal sprouting was assessed by immunohistochemistry against Anti-Growth Associated Protein-43 (GAP-43), which stains sprouting axons. GAP43 was abundantly expressed in urethral sections from intact (**A**), 1-week (**B**) and 4-weeks post-SCI animals (**C**). The co-localization of GAP-43 with all markers of nerve fibres was observed (**D**–**H**), irrespective of the time point of disease progression and location within the urethral wall. We found no significant differences in GAP-43 content (**I**), irrespective of the time point of disease progression. Scale bars equal 50 µm. The quantification of labelling intensity by Image J in the extension of the proximal urethra (n = 4). One-way ANOVA followed by Tukey's multiple comparison test.

3. Discussion

The present study investigated tissue reorganization in the urethra associated with urinary dysfunction caused by SCI. We observed profound histological changes, including a marked atrophy of the IUS. In addition, we also found evidence of a loss of sensory and sympathetic fibres of the urethral wall, possibly contributing to SCI-induced urinary impairment. To our knowledge, this study provides the first evidence of time-dependent histologic and innervations changes taking place in the urethra wall following SCI.

Here, a transection-type rat model of SCI was chosen, and a complete spinal transection at the T8-T9 level was performed. LUT function was analysed under urethane anaesthesia. As before [26], bladder reflex activity was strongly reduced 1 week after SCI, consistent with spinal shock [4]. Four weeks post-SCI, the frequency and amplitude of bladder contractions was markedly increased, indicating that NDO was already established. An analysis of haematoxylin/eosin-stained urethral sections showed that spinal intact animals presented a stratified epithelium. One week post-SCI, a marked disorganization was observed in the epithelium lining the proximal urethra, with signs of desquamation in the superficial layers. These results are in line with studies performed in bladders from SCI rats in acute stages of the disease, reporting alterations in epithelial morphology, including a loss of apical cells, general disorganization, and a reduction in cellular volume [17,27]. At 4 weeks post-SCI, urethral epithelial layers were again evident, with fewer signs of cellular loss and a higher number of superficial flattened cells. The epithelium height had significantly increased when compared with intact animals. These changes are similar to those observed in bladder sections obtained from SCI animals at the same time point. Indeed, 4 weeks after SCI, the bladder epithelium had recovered its organization, but the expression of differentiation markers indicated incomplete regeneration [27]. Although not explored in the present work, one could speculate about the occurrence of a similar process in the urethra. The reasons explaining the high cellular turnover in the LUT epithelium, including at the urethra, may result from the exposure to high levels of catecholamines, such as norepinephrine, released in the bladder following acute SCI [17] but likely not maintained in chronic stages of disease progression.

The urethral sphincter is the functional contractile unit of the urethra; it is responsible for the control of the bladder neck open and, therefore, urine expulsion. The internal layer of the sphincter, the IUS, is composed of smooth muscle fibres extending from the bladder body and controlled by the autonomic nervous system. The external layer of the sphincter comprises striated muscle fibres operating under the activity of the somatic nervous system. As in the urethral mucosa, changes in the urethral sphincter were also found, including signs of fibrosis in the EUS but not in the IUS. We also observed marked muscle atrophy of the IUS, as evidenced by a significant decreased expression of SMA at 4 weeks post-SCI. This is in contrast with observations performed in the bladder. Previous studies reported muscle hypertrophy [16] and changes in orientation of the detrusor smooth muscle fibres [28], accompanied by signs of fibrosis [16,29]. These morphological changes in the bladder may reflect adjustments linked to organ enlargement [28] and are considered part of the compensatory mechanisms operating to adjust this organ to the increased mechanical demands on the bladder wall due to prolonged periods of high intravesical pressure [28]. As the urethra is not the urine reservoir and thus does not suffer increases in pressure and distention during storage, it is possible that, unlike the detrusor, the IUS becomes atrophic and does not increase collagen content. Fibrosis was only seen in EUS, suggesting that the occurrence of tissue rearrangement likely does not affect the volume of striated muscle cells, as the transverse area of these cells was not altered by SCI.

The consequences of SCI in the urethral innervation were also assessed by analyzing the expression of the pan-neuronal marker β-III tubulin. We found signs of denervation, and by using specific antibodies, it was possible to identify a significant loss of sensory (mucosa, IUS, and EUS) and sympathetic (IUS) fibres, but no changes in parasympathetic innervation were found. While the distribution of peptidergic fibres in the bladder of intact and SCI individuals is well described [18], to our knowledge, the same does not apply to the urethra after SCI. Like others [30], in spinal intact animals, we identified a suburothelial sensory nerve plexus extending into the urethral epithelium. In the muscular layer, CGRP-positive afferents were also present in the IUS, with some CGRP-positive fibres also in the EUS. Therefore, the urethral sensory innervation follows a similar distribution as in the bladder, where CGRP-positive fibres were described in the urothelium [31], in the supporting lamina propria, and close to smooth muscle cells. Urethral CGRP expression was strongly impacted by SCI, with a marked decrease being observed at 1 week post-SCI

in both the mucosa and muscular layer. This is in contrast with observations made in the bladder, as previous studies demonstrated the post-SCI sprouting of sensory afferents [26], which also occurred in the neuronal pathways regulating LUT function [25,32,33]. This impairment of the sensory innervation of the urethra may contribute to SCI-induced detrusor-sphincter dyssynergia. In mice, it has been demonstrated that the relaxation of the urethral sphincter during voiding is regulated by capsaicin-sensitive C-fibre bladder afferents [34]. While yet to be demonstrated, one could speculate that a reduction in the population of urethral peptidergic afferents, most likely sensitive to capsaicin, as in the bladder [31], should impair sphincter relaxation and contribute to the loss of coordination between detrusor contraction and the relaxation of the urethral sphincter.

Sympathetic innervation of the urethra was analysed by assessing the expression of TH. These fibres are critical in promoting urine storage, as they induce sphincter contraction via the release of norepinephrine while relaxing the bladder body [35]. In spinal intact animals, TH-positive fibres were predominantly observed in the IUS and were notably absent in the mucosa. SCI animals presented a marked decrease in sympathetic innervation, observed as early as 1-week post-lesion and maintained at 4 weeks post-SCI, when LUT function was clearly impaired. Together with the loss of urethral sensory innervation, the sympathetic denervation of the IUS could further contribute to aberrant sphincter activity and LUT dysfunction.

Interestingly, the lack of sympathetic input may help to explain IUS atrophy. One should remember that, in other systems, including blood vessels, it has been demonstrated that norepinephrine has a trophic effect on smooth muscle cells [36,37]. It is possible the same could happen in the urethra, where a lack of sympathetic input could have resulted in the IUS atrophy of smooth muscle cells. Importantly, smooth muscle cells are established sources of Nerve Growth Factor (NGF) in the LUT, releasing this neurotrophin upon stretching [38,39]. As peptidergic afferents, including those present in the urinary bladder, are highly dependent on NGF [40], one could hypothesize that a lack of sympathetic input in the urethra may lead to IUS atrophy, triggering a reduction in NGF tissue levels, which, in turn, could lead to a loss of CGRP-positive fibers due to NGF deprivation. A reduction in NGF could further accentuate sympathetic loss, as sympathetic neurons are also dependent on this neurotrophin for survival [41]. The reasons, however, for sympathetic denervation remain unclear, and it is difficult to pinpoint the initial trigger for this dysregulation.

In the bladder, parasympathetic innervation is particularly important in the voiding phase. During storage, these circuits are silent and are activated only when the bladder reaches its maximum capacity to promote detrusor contraction for urine expulsion [42]. In contrast, in the urethra, parasympathetic innervation has an inhibitory effect mediated by nitric oxide release, resulting in sphincter relaxation. Intact animals presented positive VAChT-positive fibres in suburothelial layers. In some cases, some parasympathetic fibres extended their processes into the urothelium, as previously observed by other investigators [30]. Parasympathetic fibres were also present in the IUS, but very few were observed in the EUS. The analysis of urethral sections obtained from SCI animals, irrespective of the time point, did not show any significant differences when compared to intact animals. This is in contrast with observations made in the bladder of SCI animals. In this case, the parasympathetic innervation of the detrusor was the most affected by SCI, with studies demonstrating that VAChT fibres decreased in the first days after SCI, partially recovering with the time course but failing to reach basal levels [43]. The reasons why urethral parasympathetic innervation was not affected by SCI remain elusive.

Because axonal expansion is known to be a key mechanism for NDO emergence [25,26,32], sprouting was also studied by assessing the expression of GAP-43, an established marker of axonal growth [44]. We found the co-localization of GAP-43 with all the nerve fibre markers studied, suggesting that all populations of nerve fibres coursing in the urethral wall undergo some degree of morphological plasticity. The quantification of GAP-43 expression showed no significant changes after SCI, contrasting to what is known to occur in the bladder and the neuronal pathways involved in micturition control [26,32].

While the sprouting of bladder afferents has been linked to high levels of NGF in the bladder [45], the concentration of NGF in the urethra wall should likely have decreased as a result of IUS atrophy, considering that smooth muscle cells are important sources of NGF [38,39]. Therefore, no abnormal axonal sprouting was observed in the urethra of SCI rats in comparison with spinal intact animals.

4. Materials and Methods

4.1. Animals and Drugs

The experiments were performed using in-house bred, 200–250 g Wistar rats, derived from the Charles River Laboratories (France) colony. Female rats were preferred considering the wealth of published data on urinary impairment after SCI using these animals. Moreover, female rats were also preferred because it is easier to accomplish urine removal during the bladder areflexia period. The animals were maintained under a 12 h light/dark schedule, with free access to food and water. Spinal cord injuries were performed under deep anesthesia induced by the intraperitoneal injection of ketamine (60 mg/kg) and medetomidine (0.25mg/kg). Anaesthesia was reverted with atipamezole (1 mg/kg), delivered as an intramuscular injection. For cystometries, the animals received a subcutaneous injection of urethane (1.2 g/kg), followed by sodium pentobarbital (400 mg/kg), prior to euthanasia. The antibodies used in the immunohistochemistry experiments are indicated in Tables 1 and 2.

Table 2. This table depicts the list of primary antibodies used in the present study. The source, host species, and dilution are indicated.

Primary Target	Dilution	Host Species	Manufacturer	Reference
SMA	1:1000	rabbit	Abcam	Ab124964
B-III tubulin	1:1000	rabbit	Synaptic systems	Sysy302302
GAP43	1:500	sheep	Novus	NBP1-41123
CGRP	1:1000	rabbit	Cell signaling	14959
VachT	1:1000	rabbit	Synaptic systems	Sysy139103
TH	1:500	rabbit	Abcam	Ab137869

The experimental procedures were carried out according to the European Communities Council Directive 2010/63/EU and local regulations (protocol ORBEA_56_2017/1218; 31 January 2019). All efforts were made to reduce the number of animals used as well as to reduce animal stress and suffering.

4.2. Complete Spinal Cord Transection

After anesthesia and laminectomy between the T7-T10 vertebrae, the T8/T9 spinal segments were exposed for complete sectioning. A small piece of sterile haemostatic sponge was inserted between the retracted cord ends to limit bleeding. During the recovery period, the animals received oral antibiotics for 8 days (enrofloxacin 5 mg/kg), and, if necessary, they received analgesic drugs (tramadol 0.4 mg/kg). In order to avoid urinary retention, bladders were manually emptied daily by abdominal compression until automatic voiding was established. The experimental groups (n = 4–6 animals per group) included animals left to recover 1 (1 wk SCI) and 4 weeks (4 wks SCI) following the spinal lesion. Spinal intact rats (INT) were used as controls.

4.3. Cystometries and Terminal Handling

In order to evaluate the bladder reflex activity, the animals were submitted to cystometry under urethane anesthesia. The animals were placed in a heating plate to maintain body temperature at 37 °C. Following deep anesthesia, a suprapubic skin incision was made, and muscle bundles were separated for bladder exposure. A 21-gauge needle was

inserted into the bladder dome, and sterile saline was infused for 1 h at a rate of 6 mL/h. Bladder contractions were recorded by a pression transducer connected to the needle. After cystometry, animals were euthanized with an intraperitoneal injection of sodium pentobarbital. Cystometrograms were analyzed using LabScribe software 2.0 (World Precision Instruments, Hertfordshire, UK).

4.4. Histological Analysis

After euthanasia, urethras were dissected, collected, and fixed overnight in 4% formalin solution. This was followed by sequential dehydration with different concentrations of ethanol and benzene before paraffin impregnation. Serial longitudinal 5 µm urethral sections were obtained in a Leica RM2145 microtome and collected in poly-L-lysine coated slides. For routine histological staining, sections were deparaffinised with benzene and gradually rehydrated with ethanol solutions. Haematoxylin–eosin staining was used to analyse tissue histology, and Sirius Red staining was used to assess collagen content as a measure of fibrosis levels. Images were obtained using a Carl Zeiss microscope equipped with a CH-9435 Leica camera. The Leica application suite (VAS 4.6.0) software was used for image capturing.

4.5. Immunohistochemical Tissue Assessment

Urethras were collected and fixed in 4% paraformaldehyde solution for 6 h before cryoprotection in 30% sucrose solution with 0.1% sodium azide for 24 h. Longitudinal urethral sections (14 µm thick) were obtained in a freezing cryostat, collected in Superfrost Plus slides, and stored at −20° until further processing.

The expression of β-III tubulin (a general marker of nerve fibres) was assessed using the ABC method for light microscopy. Briefly, after several washes with a phosphate-buffered saline solution (PBS), sections were incubated in 0.3% hydrogen peroxide solution in PBS to inhibit endogenous peroxidase. After subsequent washes, sections were blocked for 2 h with 10% normal horse serum in PBS containing 0.3% Triton X-100 (PBST) followed by a 48 h incubation at 4 °C with anti- β-III tubulin antibody. After several washes with PBST, sections were incubated with a biotinylated swine anti-rabbit antibody for 1 h. The immunoreaction was visualized using the ABC peroxidase-conjugated method (1:200; Vectorlabs, UK), using 3,3′-diaminobenzidine tetrahydrochloride (5 min in 0.05 M Tris buffer, pH 7.4, containing 0.05% DAB and 0.003% hydrogen peroxide) as chromogen. Sections were cleared in xylene, cover-slipped, and observed. All antibodies and the ABC complex were prepared in PBST.

Immunofluorescence techniques were used to analyse the expression of smooth muscle actin (SMA) and nerve fibre markers, including calcitonin gene-related peptide (CGRP), tyrosine hydroxylase (TH), and vesicular acetylcholine transporter (VAChT). To assess axonal sprouting in the urethra wall, an antibody against GAP43, a marker of axonal growth [44], was used in combination with the antibodies mentioned above. SMA levels were detected in paraffin-embedded sections, which were de-paraffinized, re-hydrated, and submitted to antigen retrieval using citrate buffer boiling.

In all cases, the slides were washed with PBST and blocked with 10% of normal horse serum (NHS) in PBST for 1 h at room temperature (RT). The sections were then incubated for 48 h at 4 °C with a primary antibody (Table 2), after which they were thoroughly washed and incubated for 1 h in a mixture of fluorescently labeled secondary antibodies (Table 3). Concentrations of primary and secondary antibodies are indicated in Tables 2 and 3. Finally, sections were mounted using an anti-fade mounting medium (Thermo Fisher Scientific, Rockford, IL, USA) and observed in a fluorescence Zeiss microscope (Axioimager Z1, Zeiss Z1 from Zeiss) using the AxioVision 4.6 software.

Table 3. This table depicts the list of secondary antibodies used in the present study. The source, host species, and dilution are indicated.

Secondary Target	Dilution	Host Species	Manufacturer	Reference
Rabbit/Alexa 488	1:1000	donkey	ThermoFisher	Ab21206
Rabbit/biotinylated	1:200	swine	Dako Denmark	E0353
Sheep/Alexa 568	1:1000	goat	ThermoFisher	A21206

4.6. Quantifications and Statistics

Cystometograms were analyzed using the LabScribe software (version 2.34900; iWorx Systems, Friedberg, Germany). Only bladder contractions with amplitudes superior to 10 cm H_2O were quantified.

All image data were collected in the proximal urethra, both in the posterior (close to the vagina) and anterior side and quantified using the Image J software. The thickness of the urethral epithelium and lamina propria was measured as an average of six measurements per animal, as indicated in Figure 2A. Image J was also used to determine the collagen staining area and the area of EUS muscle fibres, measured in transversal individual sections. Labelling intensity was determined by densitometry and obtained in three regions of interest: mucosa, IUS, and EUS. In all cases, data are presented as an average of the values measured in both sides of the urethra ± standard deviation.

All statistical analyses were conducted using GraphPad 9.0 software. Datasets were first tested for normality (Shapiro–Wilk normality test), followed by variance analysis testing using one-way ANOVA followed by Tukey's post hoc test. In all cases, $p < 0.05$ was considered statistically significant. The data are presented as the mean ± standard deviation (SD).

5. Conclusions

While many investigators have focused on the bladder, to our knowledge, this is the first study providing evidence of profound tissue reorganization in the urethra after SCI. We found signs of epithelial damage and recovery, accompanied by IUS atrophy, and marked sensory and sympathetic denervation. These changes likely contribute to the urinary impairment that follows SCI. This should be confirmed in future studies aiming to further unravel the pathophysiological mechanism of SCI-induced urinary dysfunction, paving the way for therapies exclusively targeting the urethra.

Author Contributions: All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by A.F. and S.S.C. The first draft of the manuscript was written by A.F., and all authors commented on previous versions of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Experiments were conducted under approval of the Animal Ethical Committee of the Faculty of Medicine, University of Porto (ORBEA_56_2017/1218) and of the Portuguese National Authority for Animal Health.

Conflicts of Interest: The authors declare that they have no conflict of interest.

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Publication IV

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Development of urinary impairment after Spinal Cord Injury reflects altered urethral serotonin signalling: an experimental study in female mice

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KEY WORDS

Urethra, Serotonin, Paraneurons, Spinal Cord Injury, Neurogenic Detrusor Overactivity

ABSTRACT

Spinal Cord Injury (SCI) often leads to urinary incontinence. While alterations in the bladder have been considered the primary cause of post-SCI urinary dysfunction, evidence suggests that urethral afferents may influence post-SCI bladder activity. These afferents respond to locally produced serotonin (5-HT), and 5-HT signalling may likely be affected by SCI. Here, we investigated the role of urethral 5-HT on post-SCI bladder reflex activity using female C57BL/6 mice (wild-type [WT] and animals lacking urethral 5-HT (tryptophan hydroxylase 1 [tph1] null [*tph1*$^{-/-}$]). In WT animals, bladder overactivity and increased 5-HT^{+} urethral cells were observed at one and four weeks post-SCI (1w and 4w, respectively), while, in contrast, *tph1*$^{-/-}$ mice showed some preservation of bladder function. Both genotypes developed urethral smooth muscle atrophy 4w post-SCI, but fibrosis of striated muscle was detected only in *tph1*$^{-/-}$ 4w SCI mice. Sensory and cholinergic fibres were upregulated only in WT SCI mice, suggesting 5-HT depletion may block their expansion. Pharmacological blockade of 5-HT receptors did not fully prevent urinary impairment but affected SCI-induced bladder dysfunction. These observations indicate that urethral 5-HT contributes to SCI-induced urinary impairment and urethral tissue reorganization, offering new insights into the urethrovesical reflex and potentially pinpointing new therapeutic targets.

1. INTRODUCTION

Appropriate urine storage and release are dependent on the coordinated contraction and relaxation of the lower urinary tract (LUT) organs- the urinary bladder (the urine reservoir), and the urethra, considered the bladder outlet and encompassing the urethral passage and sphincters [1]. The synchronised activity between the bladder and the urethra is controlled by intricate neuronal circuits, including sensory afferent pathways, and autonomic and motor circuits that mediate the correct communication between the LUT and supraspinal structures. These circuits are severely disturbed by spinal cord injury (SCI), leading to urinary impairment

[1, 2]. Following an initial post-SCI period of little or no bladder reflex activity [3], an alternative micturition reflex arises at the lumbosacral spinal cord functioning in the absence of supraspinal input [2, 4]. Consequently, post-SCI micturition becomes involuntary, and the presence of neurogenic detrusor overactivity (NDO) gives rise to strong, frequent and involuntary detrusor contractions, often concurrent with detrusor-sphincter dyssynergia (DSD) [5], resulting in urinary incontinence. Moreover, due to sustained, dangerously high intravesical pressures, there is considerable risk of damage to the upper urinary tract, development of autonomic dysreflexia, and recurrent urinary infections, worsening functional outcomes and patients' quality of life [2, 6].

The bladder has traditionally been seen as the main player in micturition and the central focus of urinary dysfunction after neurologic injury. Thus, bladder alterations in response to SCI are well documented [7-9], and pharmacological interventions are mainly focused on modulating bladder activity [4]. However, recent data obtained in various animal models and human patients have shown that the urethra, previously seen as a passive LUT component, is an active participant in micturition. In fact, urethral afferent input, generated by urine flow along the urethra during voiding, triggers a facilitatory ureterovesical feedback that increases voiding efficiency [10-16]. This reflex is suppressed by urethral anaesthesia, suggesting this effect must originate from urethral flow receptors [12, 13, 17]. Moreover, urethral electrical stimulation triggers detrusor contraction [18-21], evoking inhibitory or excitatory bladder reflexes depending on the location and frequency of the stimuli [21, 22].

As urethral nerve fibres present in the lamina propria or muscle layers do not reach the luminal surface, from where stimuli originate, the perception of sensory input in the urethra is likely mediated by specialised epithelial cells, intermingled with other lining cells [12]. These specialised cells are unique and present neuron-like properties, including the expression of sensory receptors and the ability to release neuromediators when stimulated [12, 23], and, thus, are often designated by paraneuronal cells. This group of cells include serotoninergic

paraneurones, which are mainly located along the urethral sphincter region, in the vicinity of sensory and cholinergic nerves [24]. The importance of serotonin (5-HT) in the regulation of an urethrovesical reflex has been shown by Coelho and coworkers. They demonstrated that, under isovolumetric cystometry, the instillation of a 5-HT solution into the urethral channel triggers robust bladder-reflex contractions. On the other hand, under normal cystometry, i.e. with an open urethral outlet, 5-HT administration decreases frequency, while strongly enhancing the amplitude of bladder contractions. Altogether, these observations support the existence of a serotonergic urethrovesical reflex that operates through complex neuronal mechanisms rather than a simple translocation of neuromediators between adjacent organs [24].

Changes in the urethra in cases of bladder dysfunction, as after SCI, have been seldom addressed, although there is abundant information about SCI-induced changes in bladder structure and function [4, 7, 8, 25, 26]. This matter was explored in a recent study, which described significant urethral tissue rearrangement in response to SCI, including smooth muscle atrophy and fibrosis in the external urethral sphincter, accompanied by loss of sensory and autonomic neuronal fibres [27]. Here, to expand this previous study and further deepen our understanding of the effects of SCI on LUT function, we focused on the role of urethral 5-HT and further investigated the effects of SCI on this organ.

2. RESULTS

2.1. *Tph1*$^{-/-}$ mice display attenuated urinary dysfunction in chronic SCI

To evaluate bladder function after SCI, animals were deeply anaesthetised with urethane and underwent cystometries before euthanasia. Urodynamic recordings from WT intact (WT INT) animals were indicative of normal bladder function, with high-amplitude and low-frequency bladder contractions and normal intravesical pressures (Figure 1A, G-J; Table 1). Intact animals lacking peripheral serotonin (*tph1*$^{-/-}$ INT) presented a similar pattern of bladder function (Figure 1B, G-J; Table 1).

One-week post-injury, bladder function was severely disrupted, with signs of bladder dysfunction in both genotypes. Compared to WT INT animals, WT 1w SCI mice showed significantly decreased amplitude of bladder reflex contractions (Figure 1C; H; Table 1; p<0.001 vs WT INT). The frequency of bladder reflex contractions presented an increased tendency, as well as the basal pressures. Peak pressures remained similar (Figure 1C, G, I-J; Table 1). Likewise, *tph1*$^{-/-}$ 1w SCI mice presented decreased amplitude of bladder contractions when compared to *tph1*$^{-/-}$ INT animals (Figure 1D-H, Table 1; p<0.001 vs *tph1*$^{-/-}$ INT), as well as a tendency for increased frequency and basal pressures that did not reach statistical significance (Figure 1D, G, I-J; Table 1).

Four weeks post-SCI, the bladder reflex activity of WT mice worsened. When compared to intact mice, the frequency of bladder contractions increased (Figure 1E, G; Table 1; p<0.001 vs WT INT), as well as the basal (Figure 1E, I; Table 1; p<0.001 vs WT INT) and peak pressures (Figure E, J; Table 1; p<0.01 vs WT 1w SCI), causing a decrease in the amplitude of bladder contractions (Figure 1E, H; Table 1; p<0.0001 vs. WT INT). At four weeks after SCI, *tph1* $^{-/-}$ mice presented a similar bladder behaviour to that observed at 1 week after SCI, except for a significantly increased frequency of bladder contractions in comparison with spinal intact *tph1* $^{-/-}$ animals (Figure 1E-F, G, Table 1; p<0.01 vs *tph1* $^{-/-}$ INT). When compared to WT counterparts at the same time-point, *tph1*$^{-/-}$ 4w SCI animals presented a significantly decreased frequency of bladder contractions (Figure 1E-G; Table 1; p<0.05 vs WT INT), basal (Figure 1E-F, I; Table 1; p<0.01 vs WT 4w SCI) and peak pressures (Figure 1E-F, J; Table 1; p<0.01 vs WT 4w SCI), suggesting an influence of 5-HT absence on NDO symptoms 4 weeks post-injury.

Table 1. Urodynamic parameters from WT and *tph1*$^{-/-}$mice submitted to spinal cord transection and left spinal intact. Abbreviations: WT, Wyld-Type; *tph1*$^{-/-}$, Homozygote *tryptophan hydroxylase 1* deficient mice; SCI, spinal cord injury. ****p≤0.0001 vs. WT INT, #p≤0.05 vs WT 1w SCI, ##p≤0.01 vs WT 1w SCI; ****p≤0.05 vs *tph1*$^{-/-}$ INT; **$**p≤0.05 vs WT 4w SCI; **$$**≤0.01 vs WT 4w SCI. Data are presented as mean ± standard deviation.

	Frequency of bladder reflex contractions (contractions per min)	**Voiding amplitude** (cm H_2O)	**Basal pressure** (cm H_2O)	**Peak pressure** (cm H_2O)
WT INT	0.4 ± 0.12	37.5 ± 1.7	8.54 ± 0.85	46.03 ± 2.3
***tph1*$^{-/-}$ INT**	0.4 ± 0.15	34.81 ± 6.57	11.35 ± 6.73	46.02 ± 4.77
WT 1w SCI	1.80 ± 0.30	14.96 ± 4.41 **** vs WT INT	22.56 ± 6.16	36.98 ± 3.54
***tph1*$^{-/-}$ 1w SCI**	1.34 ± 0.36	10.81 ± 1.85 **** vs *tph1*$^{-/-}$ INT	26.94 ± 3.6	37.64 ± 3.60
WT 4w SCI	3.65 ± 2.27 **** vs WT INT # vs WT 1w SCI	13.87 ± 2.22 **** vs WT INT	48.42 ± 24.82 **** vs WT INT ## vs WT 1w SCI	57.11 ± 22.1 ## vs WT 1w SCI
***tph1*$^{-/-}$ 4w SCI**	1.96 ± 0.83 ** vs *tph1*$^{-/-}$ INT $ vs WT 4w SCI	14.15 ± 10.8 **** vs *tph1*$^{-/-}$ INT	24.64 ± 0.85 $$ vs WT 4w SCI	41.01 ± 12.66 $$ vs WT 4w SCI

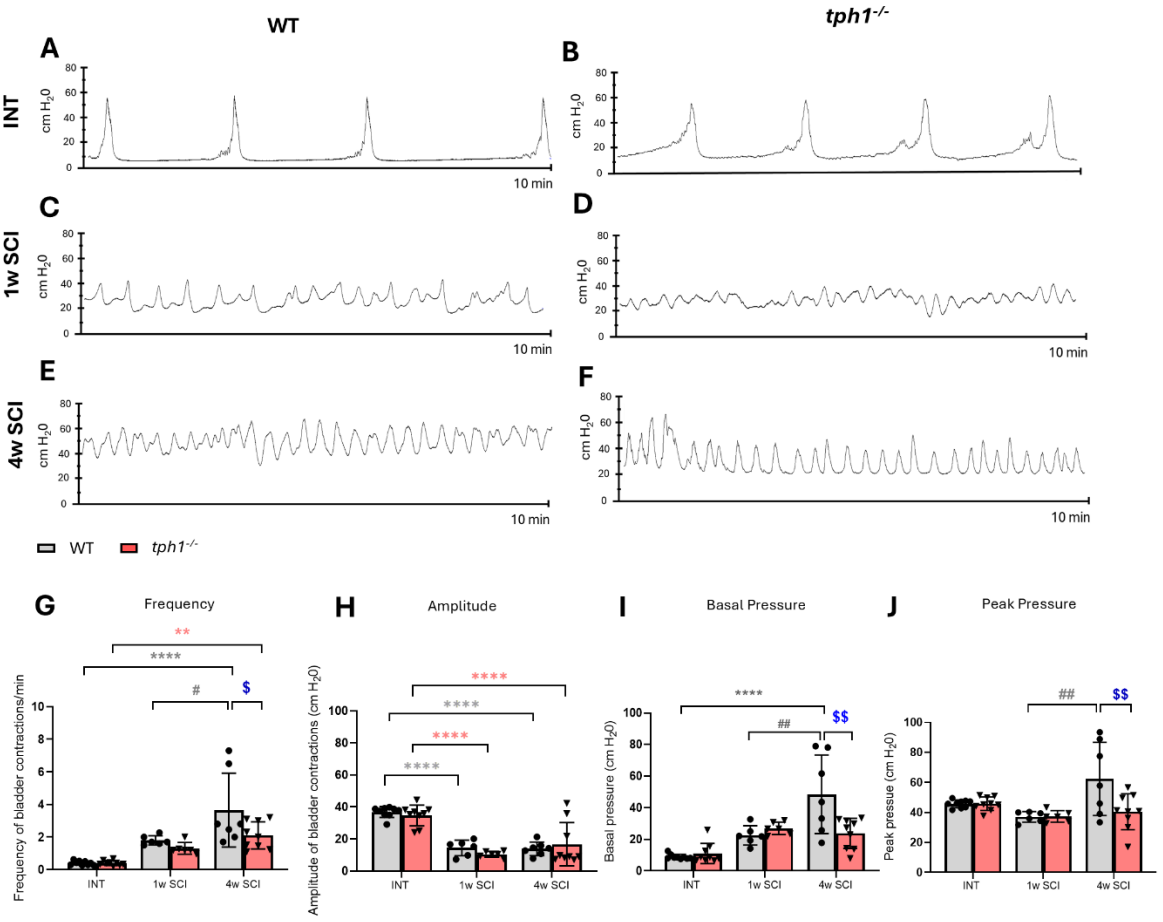

Figure 1. Effects of SCI and peripheral serotonin on urinary dysfunction. **(A-F)** Bladder function was assessed by cystometry under urethane anaesthesia. Representative cystometrograms depicting the voiding function of **(A)** Wild-type (WT) intact (INT), **(B)** *tph1*$^{-/-}$ INT, **(C)** WT one week after injury (1w SCI), **(D)** *tph1*$^{-/-}$ 1w SCI, **(E)** WT four weeks after injury (4w SCI), and **(F)** *tph1*$^{-/-}$ 4w SCI mice; **(G-J)** Analysis of urodynamic parameters of **(G)** frequency of bladder contractions, **(H)** amplitude of bladder contractions, **(I)** basal pressure, and **(J)** peak pressure. INT animals from both genotypes presented typical bladder function (A-B, G-J), while both WT and *tph1*$^{-/-}$ SCI mice showed signs of urinary dysfunction at 1w SCI (C-D, G-J). Four weeks after injury, urinary dysfunction was maintained in both genotypes, but *tph1*$^{-/-}$ mice presented attenuated signs of urinary dysfunction, compared to WT 4w SCI animals (F, G-J). Graphs represent mean ± standard deviation, and $p<0.05$ was considered statistically significant. ****$p<0.0001$ vs WT INT; **$p<0.001$ vs *tph1*$^{-/-}$ INT; ****$p<0.0001$ vs *tph1*$^{-/-}$ INT; #$p<0.05$ vs WT 1w SCI; ## $p<0.01$ vs WT 1w SCI; $$p<0.05$ vs WT 4w SCI; $$$p<0.01$ vs WT 4w SCI. Two-way ANOVA followed by Tukey´s post-hoc multiple comparison test.

2.2. Development of SCI-induced urinary dysfunction courses with upregulation of urethral serotonergic paraneurons

To study the presence of serotonergic paraneurons in the urethral lining of WT and *tph1*$^{-/-}$ mice following SCI, an immunofluorescence staining against 5-HT was performed. In WT mice, immunoreactive cells were slender and elongated in shape, located in the deeper layers of the epithelium and exhibiting thin processes towards the luminal surface or lamina propria (Figure 2A-C). The number of 5-HT^{+} cells increased in response to SCI in a time-dependent manner. This increase was already apparent one week after injury (WT 1w SCI), but only reached statistical significance four weeks later, when the number of 5-HT^{+} cells tripled in comparison to intact WT mice (Figure 2B; $p<0.01$ vs WT INT). These serotonergic cells were located near sensory peptidergic neuronal fibres, labelled with anti-calcitonin gene-related peptide (CGRP) antibody (Figure 2D-F). No 5-HT^{+} cells were detected in urethral sections from *tph1*$^{-/-}$ mice, whether they were spinal intact or submitted to SCI (Figure 2G-I).

Central expression of 5-HT was determined by HPLC in the lumbosacral spinal cord (L5-S1 spinal cord segments). After SCI, 5-HT levels significantly decreased in both WT and *tph1*$^{-/-}$ mice,

both at 1 week and 4 weeks post-SCI, demonstrating the disruption of central 5-HT pathways induced by SCI (Figure 2K; p<0.0001 vs INT for both genotypes).

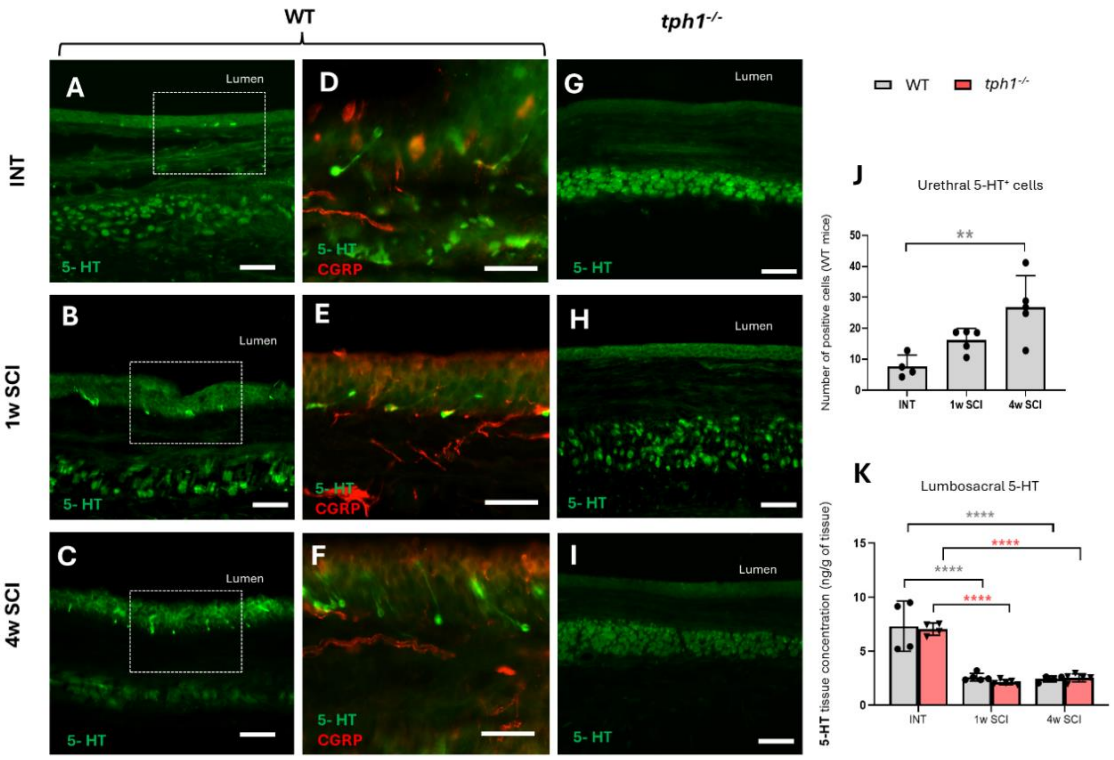

Figure 2. Effects of SCI on the peripheral and central 5-HT production. The presence of serotonin (5-HT) positive cells in the urethral epithelium was assessed by immunostaining against 5-HT. Central concentration of 5-HT was measured by HPLC in the lumbosacral spinal cord (L5-S1 spinal cord segments). (**A-C**) Representative images of 5-HT staining in WT **(A)** INT, **(B)** 1w SCI and **(C)** 4w SCI animals. (**D-F**) Magnification of urethral double-immunostaining against 5-HT and calcitonin gene-related peptide (CGRP) (represented by dashed boxes in A-C) in WT (**D**) INT, (**E**) 1w SCI and (**F**) 4w SCI, showing the proximity of 5-HT^{+} cells to sensory peptidergic fibres; (**G-I**) Immunostaining against 5-HT in the urethral epithelium of *tph1*$^{-/-}$ (**G**) INT, (**H**) 1w SCI and (**I**) 4w SCI mice confirming the absence of 5-HT^{+} cells in *tph1*$^{-/-}$ mice. (**J**) Quantification of 5-HT^{+} cells in WT animals, showing a significant increase in the number of 5-HT-stained paraneurons at 4w SCI. (**K**) HPLC quantification of 5-HT concentration in the lumbosacral spinal cord in WT and *tph1*$^{-/-}$ INT, 1w SCI and 4 w SCI animals showed a significant decrease of this neurotransmitter 1 and 4 weeks after injury. Graphs represent mean ± standard deviation, and p<0.05 was considered statistically significant. **p<0.01 vs WT INT; ****p<0.0001 vs WT INT; ****p<0.0001 vs *tph1*$^{-/-}$ INT. One (graph J) or two-way ANOVA (graph K) followed by Tukey´s post-hoc multiple comparison test. Scale bars correspond to 50 µm.

2.3. Alterations in urethral innervation in response to SCI are dependent on peripheral 5-HT synthesis

As before [24], the distribution of sensory and cholinergic fibres, in the vicinity of cell processes of serotonergic urethral paraneurons, was investigated by immunohistochemistry in the urethra. Sensory innervation was assessed by CGRP immunostaining, an established marker of peptidergic sensory fibres. In the presence of peripheral 5-HT (WT mice), SCI induced a time-dependent increase in CGRP expression in the internal urethral sphincter (IUS) region, particularly four weeks after injury (WT 4w SCI) (Figure 3A, C; $p<0.01$ vs WT INT). No time-dependent alterations in CGRP expression were detected in the external urethral sphincter (EUS) (Figure 3A, D). In the absence of peripheral 5-HT expression (*tph1*$^{-/-}$ mice), no changes were detected in the urethral (IUS and EUS) sensory innervation after SCI, irrespective of the timepoint after spinal trauma (Figure 3B-D).

Parasympathetic innervation was assessed by immunostaining urethral sections against vesicular transporter of acetylcholine (VAChT), an established marker of cholinergic parasympathetic fibres. VAChT^{+} fibres were significantly increased in WT mice submitted to SCI at 4 weeks after injury (WT 4w SCI). This was evident at the IUS but not at the EUS region (Figure 3E, G-H; $p<0.01$ vs WT INT; $p<0.01$ vs WT 1w SCI). In the absence of peripheral 5-HT expression (*tph1*$^{-/-}$ mice), no time-dependent changes in the cholinergic innervation of the IUS and EUS after SCI were observed.

2.4. SCI-induced smooth muscle atrophy of the IUS does not depend on peripheral 5-HT

The integrity of the IUS, known to be affected after SCI [27], was evaluated by immunostaining of smooth muscle actin (SMA). The abundant expression of SMA found in intact animals significantly decreased four weeks after SCI both in WT (Figure 3I, K; $p<0.05$ vs WT INT; $p<0.01$ vs WT 1w SCI) and *tph1*$^{-/-}$ mice (Figure 3J, K; $p<0.05$ vs *tph1*$^{-/-}$ INT; $p<0.001$ vs *tph1*$^{-/-}$ 1w SCI), demonstrating signs of IUS atrophy independent of peripheral 5-HT.

2.5. Striated muscle fibrosis is dependent on peripheral 5-HT synthesis

The collagen content of the EUS was measured by Sirius-Red staining to assess fibrosis. In WT animals, collagen areas remained unchanged by SCI progression, with a slight but not significant decrease at 1w SCI, which returned to baseline values at 4w SCI (Figure 3L, N; $p<0.01$ vs WT 1w SCI). On the other hand, *tph1*$^{-/-}$ mice displayed a time-dependent increase in EUS collagen content. At 4w SCI, collagen levels were significantly higher in *tph1*$^{-/-}$ mice compared to intact counterparts (Figure 3L-M; $p<0.05$ vs INT *tph1*$^{-/-}$). Although not statistically significant, EUS collagen content in *tph1*$^{-/-}$ 4w SCI appeared to increase, compared to WT animals at the same timepoint.

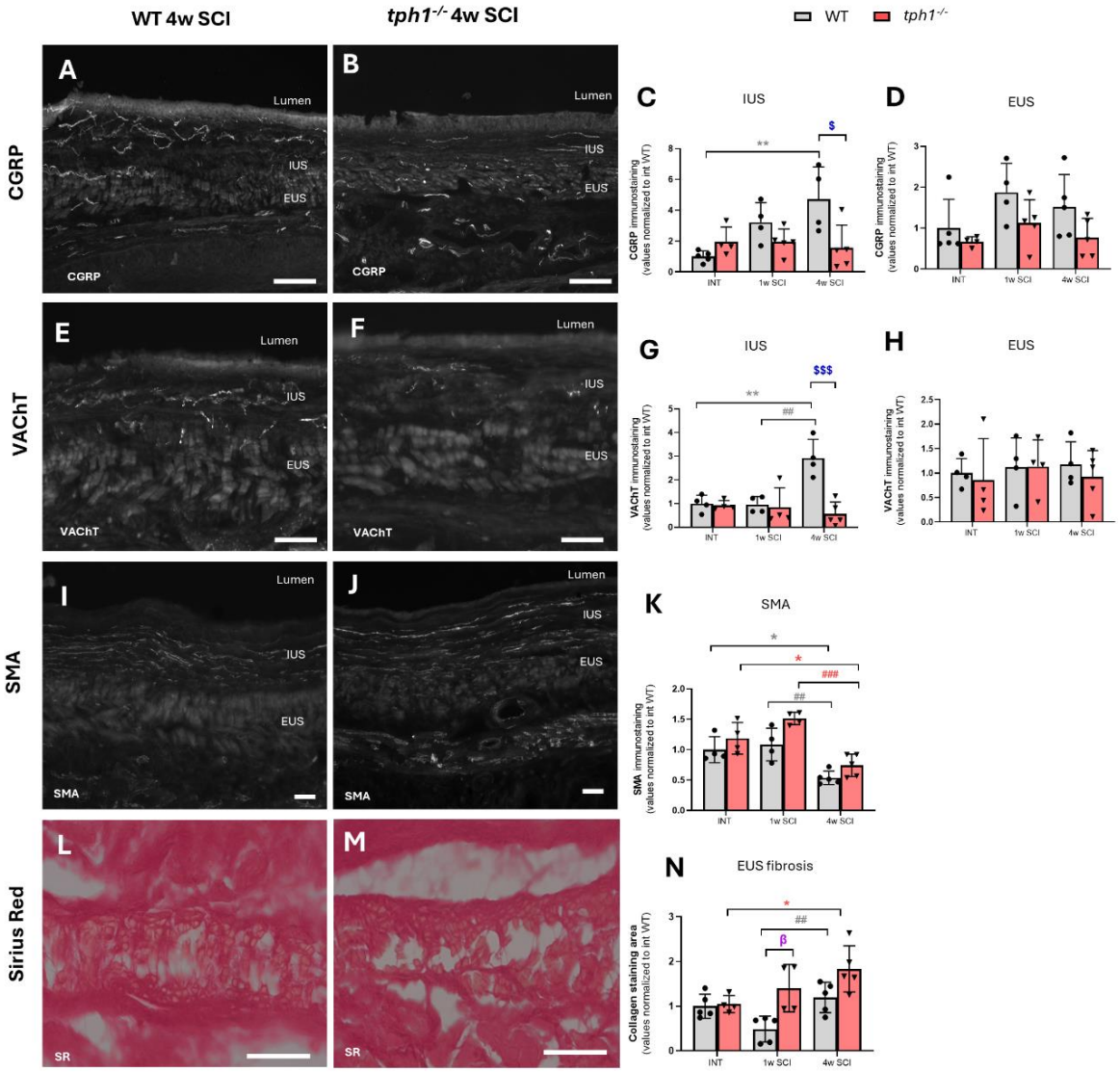

Figure 3. Influence of peripheral serotonin on urethral tissue rearrangement after SCI. (**A-D**) Sensory innervation assessment by calcitonin gene-related peptide (CGRP) staining in the proximal urethra. Representative images of (**A**) WT 4w SCI and (**B**) *tph1*$^{-/-}$ 4w SCI mice CGRP staining. Densitometry quantification of sections of the (**C**) internal urethral sphincter (IUS) showed significantly decreased CGRP^{+} fibres 4 weeks after injury in *tph1*$^{-/-}$ mice, compared to WT animals. No time-dependent changes in CGRP^{+} fibres were seen in the (**D**) external urethral sphincter (EUS), independently of the genotype. (**E-H**) Parasympathetic innervation assessment by vesicular transporter of acetylcholine (VAChT) staining in the proximal urethra. Representative images of (**E**) WT 4w SCI and (**F**) *tph1*$^{-/-}$ 4w SCI mice VAChT staining. Quantification in the (**G**) IUS showed decreased VAChT sprouting 4 weeks after injury in *tph1*$^{-/-}$mice, compared to WT at the same timepoint. No time-dependent changes in VAChT^{+} fibres were seen in the (**H**) external urethral sphincter (EUS), independently of the genotype. (**I-K**) Smooth muscle cells' integrity assessment by smooth muscle actin (SMA) immunoreaction in the urethra. Representative images of (**I**) WT 4w SCI and (**J**) *tph1*$^{-/-}$ 4w SCI mice SMA staining. (**K**) Quantification showed significantly decreased SMA expression 4 weeks after SCI, regardless of the genotype. (**L-N**) Collagen deposition in EUS cells was assessed by Sirius Red (SR) Staining. Representative images of (**L**) WT 4w SCI and (**N**) *tph1*$^{-/-}$ 4w SCI mice SR staining. (**N**) In WT animals, collagen areas remained unchanged by SCI progression, but in the absence of peripheral 5-HT, EUS fibrosis tended to increase in a time-dependent manner, reaching statistical significance at 4w SCI when compared to intact. Graphs represent mean ± standard deviation, and $p<0.05$ was considered statistically significant. *$p<0.05$ vs INT WT; **$p<0.01$ vs INT WT; ##$p<0.01$ vs WT 1w SCI; *$p<0.05$ vs *tph1*$^{-/-}$ INT; ###$p<0.001$ vs *tph1*$^{-/-}$ 1w SCI; $p<0.05$ vs WT 4w SCI; $$$p<0.001 vs WT 4w SCI. Two-way ANOVA followed by Tukey´s post-hoc multiple comparison test. Scale bars correspond to 50 µm.

2.6. Treatment with 5-HT receptor antagonists altered urinary function but did not prevent SCI-induced NDO emergence in WT mice

To test whether modulation of urethral 5-HT signalling could improve urinary function following spinal injury, SCI WT mice were daily treated with Ritanserin and Ondansetron, antagonists of 5-HT$_2$ and 5-HT$_3$ receptors, respectively, and known to have an effect on bladder activity [24, 28]. Ritanserin and Ondansetron were intraperitoneally administered separately (1 mg/kg) or in combination (1 mg/kg + 1 mg/kg), and an additional group of animals received the vehicle solution (absolute ethanol+H_2O). Animals were submitted to daily removal of urine by abdominal compression until the end of the 28-day protocol and the volume of urine was recorded every 3 days. This record showed persistently high urine volumes in all experimental groups (Figure 4A). Ten days post-SCI, all animal groups presented a reduction in urine volumes,

slightly more evident in vehicle-treated animals, followed by an increase at later time-points. At day 22, Ritanserin-treated animals showed a drop in urine volume and ended the protocol, at 28 days post-SCI, with the lowest volume of retained urine. At day 22, Ondansetron- and Ritanserin+Ondansetron-treated animals appeared to be able to retain higher urine volumes compared to vehicle- and Ritanserin-treated mice. However, no statistically significant differences were seen between treatments at any time point (Figure 4A). Importantly, as animals were daily assessed, it was observed that in animals treated with 5-HT receptor antagonists, some urine samples were cloudy and, in repeated occasions, it was necessary to perform anaesthetised catheterisation for urine removal in Ritanserin-treated mice. In the remaining treated groups, LUT infections were less frequent and tended to resolve quickly. In any case suspect of urinary tract infection and following in-house veterinary advice, animals received antibiotics until urine samples returned to normal.

Table 2. Urodynamic parameters from 4w SCI animals treated with vehicle, Ritanserin, Ondansetron, or Ritanserin+Ondansetron. *p<0.01 vs vehicle; **p<0.01 vs RT. Data is presented as mean ± standard deviation. Abbreviations: RT - Ritanserin; ON - Ondansetron; RT+ON – Ritanserin + Ondansetron.

	Frequency of bladder reflex contractions (contractions per min)	**Contraction Amplitude** (cm H_2O)	**Basal pressure** (cm H_2O)	**Peak pressure** (cm H_2O)
Vehicle	2.54 ± 0.40	6.031 ± 3.67	28.85 ± 14.19	34.88 ± 14.06
RT (1mg/kg)	2.84 ± 1.05	17.82 ± 7.58 ** vs Vehicle	16.08 ± 4.00	27.75 ± 14.01
ON (1mg/kg)	1.77 ± 0.52	6.03 ± 2.70 ** vs RT	29.71 ± 17.54	34.04 ± 13.76
RT+ON (1mg/kg + 1mg/kg)	2.45 ± 0.21	6.57 ± 2.66 ** vs RT	24.99 ± 11.81	31.56 ± 11.02

After 4 weeks of daily pharmacological treatment, animals were anaesthetized with urethane and underwent cystometries to evaluate bladder function. The frequency and peak pressure of bladder reflex contractions were similar between groups, although the lowest basal

pressure was recorded in animals receiving Ritanserin, albeit without statistical significance (Figure 4B-I; Table 2). Urodynamic recordings showed the amplitude of bladder contractions significantly increased in Ritaserin-treated mice, compared to other treated groups (Figure 4B-E, G; Table 2; $p<0.01$ vs vehicle; $p<0.01$ vs ON; $p<0.05$ vs RT+ON.

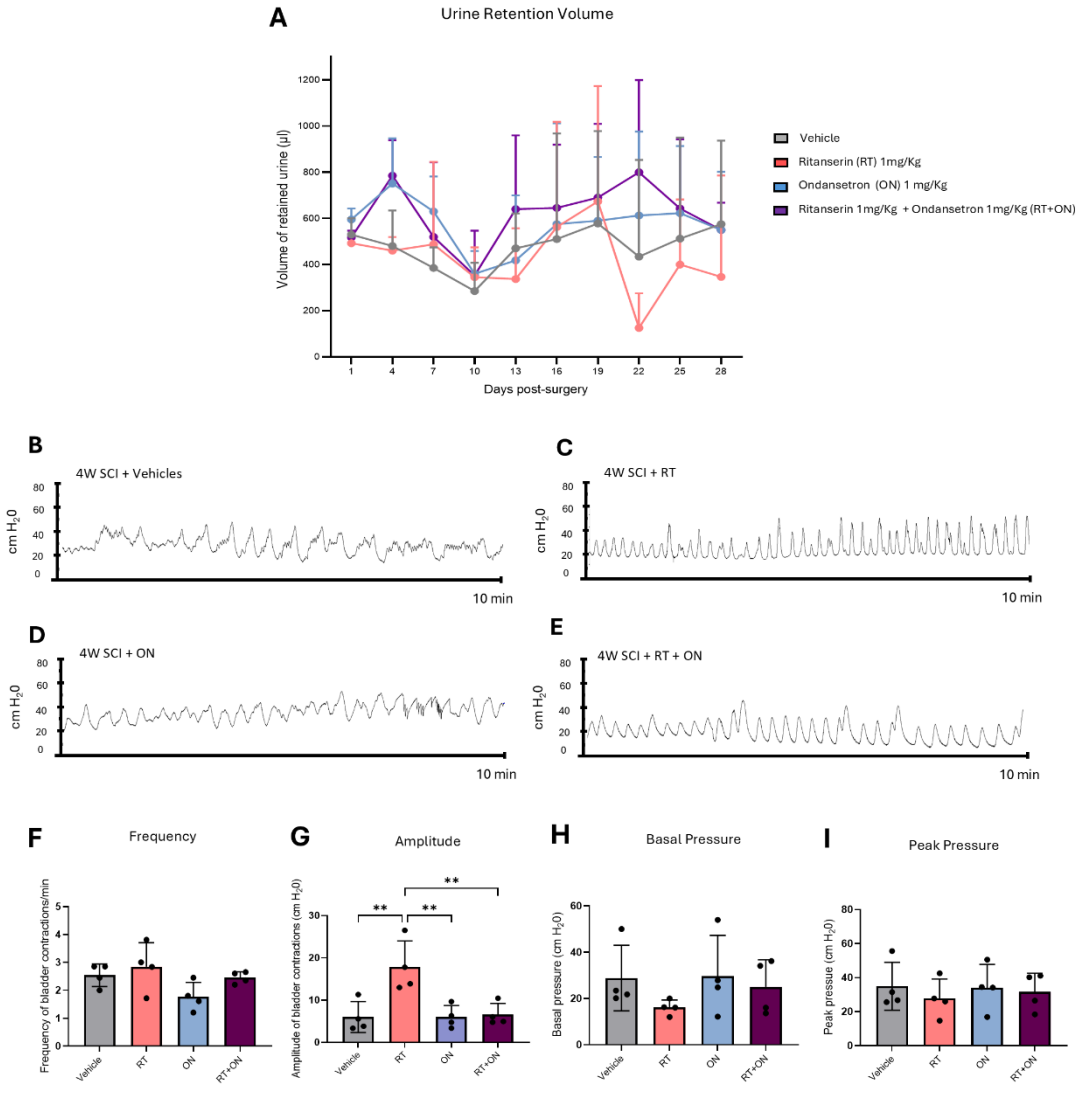

Figure 4. Effects of serotonin receptor antagonist treatment in WT 4w SCI mice. (A) Measurement of urine volume retention in 4w SCI mice receiving vehicle, Ritanserin (RT), Ondansetron (ON) or Ritanserin+Ondansetron (RT+ON). Urine volumes were measured every three days during daily manual bladder emptying. Ritanserin treatment presented a tendency for decreased urinary retention around day 20-post SCI with no statistical significance. Two-way ANOVA repeated measures analysis followed by Tukey´s post-hoc comparison test. (**B-E**) Representative cystometrograms depicting bladder contractility in mice submitted to SCI and daily-treated with (**B**) Vehicle (ethanol+H_2O), (**C**) Ritanserin (1mg/Kg), (**D**) Ondansetron (1mg/Kg) or (**E**) Ritanserin (1mg/Kg) combined with Ondansetron (1mg/Kg), for 28 days. (**F-I**) Analysis of urodynamic parameters of (**F**) frequency of expulsive bladder contractions, (**G**) amplitude of

bladder contractions, (**H**) basal pressure, and (**I**) peak pressure. Ritanserin administration alone induced significantly increased amplitudes of voiding contractions, compared to the other groups. One-way ANOVA followed by Tukey´s post-hoc comparison test (**p<0.01 vs vehicle, ON and RT+ON).

3. DISCUSSION

The present study focused on the importance of urethral 5-HT in bladder function after SCI. Experiments were performed in a mouse model of complete spinal cord transection at T8/T9 level, and the use of *tph1*$^{-/-}$ mice, which lack peripheral serotonin, allowed for a deeper understanding of the role of urethral 5-HT in LUT function.

Our results show that the bladder function of all animals, irrespective of the genotype, was affected by SCI. One week post-injury, both WT and *tph1*$^{-/-}$ mice exhibited decreased amplitude of bladder reflex contractions, accompanied by a slight, but non-significant increase in their frequency. Four weeks after injury, bladder dysfunction in WT animals was further exacerbated, with increased frequency, basal and peak pressure of bladder contractions, compared to spinal intact WT mice. In *tph1* $^{-/-}$ SCI mice, bladder function was less affected. Compared to their WT 4w SCI counterparts, *tph1*$^{-/-}$ mice presented decreased frequency of bladder contraction and basal and peak pressures, suggesting a less intense urinary dysfunction after the spinal injury became chronic. One can hypothesize that the lack of peripheral 5-HT attenuated NDO development, indicating this monoamine is involved in NDO pathophysiology, likely by modulating cholinergic and sensory fibre maladaptive sprouting. Accordingly, it has been demonstrated that 5-HT has an excitatory action on both cholinergic and sensory neurons, which amplify bladder contractility by increasing afferent drive [29-32]. As cystometric observations suggest a role for 5-HT in SCI-induced bladder dysfunction, we assessed the serotonergic population of urethral cells lining the lumen. As before, 5-HT positive cells were located in the deeper layers of the urethral epithelium, in proximity to peptidergic fibres present in the lamina propria, in parallel with what was described by Coelho and colleagues in the rat [24]. Urethral 5-HT^{+} cells were increased in WT mice 4 weeks post-SCI, likely reflecting increased

levels of this neurotransmitter, accompanying the development of NDO-like features found in cystometries. We were not able to detect the expression of 5-HT receptors in the urethra (data not shown), but high 5-HT levels likely resulted in increased excitability of bladder afferents, which is considered a key mechanism in SCI-induced urinary dysfunction [33, 34]. Heightened bladder reflex activity in SCI WT animals may be mediated via activation of 5-HT receptors [29-32], as described in animal models of interstitial cystitis/bladder pain syndrome [31, 32, 35].

Peripheral 5-HT corresponds to 98% of total body 5-HT, with its majority being produced by enterochromaffin cells in the intestinal mucosa [36]. In non-pathological conditions, peripheral 5-HT plays a minor role in peristalsis and motility, with a prominent intervention of central-derived 5-HT [37]. Only under inflammatory conditions does peripheral 5-HT assume a more prominent role, promoting a cascade of events that culminate in exaggerated gastrointestinal activity and inflammatory symptoms [38, 39]. One could speculate that a similar response may occur in the LUT, as intact animals seemed to have little dependency on peripheral 5-HT to maintain efficient urinary function. After SCI, as descending supraspinal tracts were severed, lumbosacral 5-HT was markedly reduced. This could have triggered a compensatory mechanism that increased peripheral 5-HT synthesis, eventually causing hyperexcitability of LUT afferents, which in turn enhanced bladder reflex activity and contributed to NDO and DSD.

The influence of peripheral 5-HT in sensory and cholinergic fibre rearrangement in the urethra after SCI was also studied by immunohistochemistry. Contrary to WT mice, which exhibited increased urethral expression of sensory (CGRP) and cholinergic (VAChT) markers in the IUS after SCI, levels of CGRP and VAChT in *tph1*$^{-/-}$ 4w SCI mice were similar to those observed in uninjured mice, indicating that the absence of peripheral 5-HT blocks SCI-induced expansion of sensory and cholinergic innervation in the urethral wall. As SCI lead to increased 5-HT expression in the urethral epithelium, it may be possible that high local 5-HT levels could have a direct effect on sensory and cholinergic fibres, promoting CGRP and VAChT upregulation. While a direct effect of 5-HT on the expression of these neuronal markers is yet to be demonstrated,

some studies have suggested that 5-HT can induce direct upregulation of cholinergic and sensory activity [40, 41]. Therefore, it is plausible that the absence of the monoamine in *tph1*-deficient SCI mice attenuates cholinergic and sensory markers and the responsiveness of these nerve fibres, potentially impairing neurotransmitter release.

Of note, increased urethral CGRP and VAChT expression in WT 4w SCI mice contrasts with our previous observations in the female rat, where SCI coursed with denervation of the same fibres [27]. This is probably related to differences in injury progression between rats and mice. Indeed, our urodynamic data suggest discrepancies between mouse and rat bladder reflex activity, as mice presented signs of bladder contractions at 1 week post-injury, in contrast with the SCI rat, whose bladder is mostly areflexic at this time point [27, 42]. These differences may reflect species-related differences in urethral function. For example, while rat voiding requires EUS pumping activity, mice need more prolonged periods of urethral relaxation for voiding to occur [43]. After SCI, this relaxation period is likely lost or its duration diminished, leading to inefficient voiding and urinary retention [43]. Such functional differences possibly result in different time courses of functional recovery in response to SCI, a matter requiring further investigation. Interestingly, while there was expansion of sensory and cholinergic innervation in WT but not in transgenic animals, levels of SMA were similarly decreased, suggesting smooth muscle atrophy in both genotypes. This suggests that urothelial 5-HT may be the main driver behind increased CGRP and VAChT upregulation in the IUS instead of mediators produced by smooth muscle cells, including nerve growth factor, which has a positive effect on sensory fibres [44]. The reasons for IUS atrophy remain, however, unclear.

Contrary to the rat, where SCI coursed with EUS fibrosis [27], no evidence of increased collagen content was seen in the EUS of WT 4w SCI mice. Nevertheless, this was seen in their *tph1* $^{-/-}$ counterparts, suggesting a pro-fibrotic outcome in the absence of peripheral 5-HT. This is in contrast with other studies demonstrating decreased collagen deposition in models of fibrotic syndromes in the lung, liver, and skin in spinal intact *tph1*$^{-/-}$ mice, suggesting a role of the

peripheral monoamine in collagen deposition and wound healing [45-48]. The reason for this discrepancy can only be speculated at present, but may reflect an SCI-induced peripheral immune dysregulation [49] that also occurs in the bladder [50]. As 5-HT regulates immune responses [51], an unbalanced fibrotic response may have taken place in the IUS of *tph1* $^{-/-}$ SCI mice.

Since the absence of peripheral 5-HT in *tph1*$^{-/-}$ 4w SCI led to improved bladder function, we investigated whether pharmacological blockade of 5-HT receptors involved in urethral serotonergic signalling [24, 28] could attenuate NDO symptoms in WT SCI mice. Potent and long-lasting antagonists of 5-HT_2 (Ritanserin) and 5-HT_3 (Ondansetron Hydrochloride) receptors were intraperitoneally delivered once a day for 28 days, starting on injury day. The blockade of 5-HT_2 receptor with Ritanserin lowered basal pressure and increased the amplitude of bladder contractions, potentially reflecting improved storage function after treatment. As administration of 5-HT to the urethra is known to induce strong bladder contractions [16], blockade of 5-HT_2 receptor may have reduced bladder reflex activity. In addition, while we were not able to demonstrate the effects on the activity of the sphincter, it is possible that Ritanserin could improve storage by impacting sphincter activity and promoting continence, pending on further studies to titrate the most appropriate dosage of Ritanserin. In any case, treatment with Ritanserin resulted in improved urine storage. For clinical translation, one might consider a combination of Ritanserin, to make the bladder a more effective reservoir, with clean intermittent catheterization to promote timely and periodic urine removal would mitigate risks of urinary retention and infections. Accordingly, some mice presented suggestive signs of potential urinary infections, and, upon veterinary advice, animals were maintained on antibiotic treatment.

We also tested the effects of a 5-HT_3 antagonist, Ondansetron Hydrochloride, as 5-HT_3 receptors are known to be upregulated after SCI [52, 53]. Surprisingly, Ondansetron administration did not affect bladder function as previously seen in rats [16], with no alterations

in urodynamic parameters or urine residual volumes, compared to vehicle-treated SCI mice. Interestingly, the combined treatment with Ondansetron and Ritanserin did not change bladder function in SCI mice, suggesting that 5-HT_3 blockade counteracted the beneficial effects of Ritanserin. Together, these results may indicate that in our SCI mouse model, 5-HT_2, but not 5-HT_3, may be an interesting target to modulate bladder reflex activity after SCI. However, it should be recalled that drugs were delivered intraperitoneally, making it difficult to discern the main site of action.

4. CONCLUSIONS

This study builds on prior work on urethral involvement in SCI-urinary dysfunction and on the importance of the 5-HT urethrovesical reflex. We found that SCI induced urinary impairment in WT animals that was partially prevented in *tph1*$^{-/-}$ mice. In wild-type mice, bladder dysfunction was accompanied by a time-dependent increase in serotonergic paraneurons in the urethral epithelium, with expansion of sensory and cholinergic nerve fibres in the sphincter. The upregulation of CGRP and VAChT-positive fibres was absent in *tph1*$^{-/-}$ mice. In both genotypes, there was muscle atrophy of the IUS, but EUS fibrosis was only present in *tph1*$^{-/-}$ SCI animals. Blocking the 5-HT_2 but not the 5-HT_3 serotonin receptor improved bladder reflex activity. These data suggest that modulation of peripheral 5-HT may be used as a future therapeutic tool for NDO management.

5. MATERIAL AND METHODS

5.1. Animals and drugs

In-house-bred female C57BL/6 mice (aged 4-5 months, weighing 20-25 g) were maintained under a 12 h light/dark schedule and controlled temperature and air humidity, with *ad libitum* access to food and water. Wild-type (WT) and homozygous *Tph1* deficient mice (*tph1*$^{-/-}$) were divided into three groups for each genotype: intact (INT), left to recover one week (1w SCI) or

four weeks after spinal cord transection (4w SCI) (n=4/5 animals per group). *Tph1*$^{-/-}$mice were kindly provided by Prof. Michael Bader (Max-Delbrück-Center for Molecular Medicine, Berlin-Buch, Germany). Additional groups of WT female mice were submitted to SCI and treated for 4 weeks with antagonists of 5-HT receptors: Ritanserin (1 mg/kg), Ondansetron (1 mg/kg), or a combination of the two (n=-4-5 per group). Experimental procedures were carried out following the European Communities Council Directive 2010/63/EU, as well as the ethical guidelines for the investigation in animals and the internal regulations of the Faculty of Medicine of Porto, Portugal. All efforts were made to reduce the number of animals used and their suffering.

All surgeries were performed under deep anaesthesia, induced with an intraperitoneal (IP) injection of medetomidine (1 mg/Kg) and ketamine (75 mg/Kg). Anaesthesia was reverted with an intraperitoneal injection of atipamezole (1 mg/Kg). After surgeries, animals received 5% saline-glucose (0.5 ml, subcutaneous) to compensate for blood loss and dehydration. During post-operative care, they received subcutaneous antibiotics (enrofloxacin; 5 mg/Kg) and buprenorphine (0.05 mg/Kg, twice daily). For euthanasia, animals received an IP injection of sodium pentobarbital (65 mg/g). Experimental groups were divided and processed for the collection of fresh (n=4-5) or fixed tissue (n=4-6).

5.2. Induction of spinal cord injury

Animals were submitted to a laminectomy between the T7-T10 vertebrae under deep anaesthesia. The exposed spinal cord (T8/T9) was completely sectioned with a scalpel, and a small piece of sterile haemostatic sponge was placed between the retracted ends of the cord. The surgical wound was closed in two layers and anaesthesia was reverted. Animals were placed under post-surgical observation, and post-surgical care consisted of subcutaneous administration of 0.5 ml of 5% glycosylated saline, and antibiotics and analgesics (twice daily) for 7 days. To avoid urinary retention, urine was manually drained by abdominal compression during the entire extension of the protocol.

5.3. Cystometry under urethane anaesthesia and euthanasia

Bladder reflex activity was evaluated by cystometry under anaesthesia before euthanasia in all animal groups. Following deep anaesthesia with subcutaneous urethane (1.2 g/Kg), a suprapubic skin incision was made, and muscle bundles were separated for bladder exposure. A 20-gauge needle was inserted into the bladder dome, and sterile saline was infused for 45 min at a rate of 1.6 ml/h. Bladder contractions were recorded by a pressure transducer connected to the needle. Animals were maintained on a heating plate during the procedure to conserve body temperature at 37°C. After bladder recordings, animals were euthanised with an intraperitoneal injection of sodium pentobarbital (65 mg/Kg).

5.4. Tissue perfusion and immunohistochemistry

After cystometries, animals were terminally anaesthetized and the lumbosacral spinal cord was collected and immediately frozen at -80°C. The urethras were dissected, fixed by immersion in 4% paraformaldehyde (PFA) for 6 hours, and cryoprotected in 30% sucrose in phosphate buffer with 0.1% sodium azide for at least 24 h. Collected urethras were cut into 12 µm-thick longitudinal sections in a Leica cryostat and collected in Superfrost Plus slides. Slides were stored at -20°C until further processing.

Alternate urethral sections were thawed, washed in phosphate-buffered saline (PBS) and in PBS containing 0.3% Triton 100 (PBST), and subsequently blocked with 10% normal horse serum (NHS) in PBST for 2h. Tissue sections were incubated for 72h at 4°C with primary antibodies (Table 3) in 2% NHS in PBST. After several washes with PBST, sections were incubated with suitable Alexa™ fluorochrome-labelled secondary antibody in 2% PBST (Invitrogen - ThermoFisher Scientific, Porto, Portugal) for 1h at room temperature (Table 1). After subsequent washing, sections were mounted using an anti-fade mounting medium (Slowfade® Gold Life Technologies) and observed with an epifluorescence microscope (Axioimager Z1, Ziss Z1 from Zeiss) using the AxioVision 4.6 software.

Table 3. Primary and secondary antibodies used in immunohistochemistry.

Primary antibodies	**Dilution**	**Host species**	**Manufacturer**	**Catalog #**
SMA	1:1000	rabbit	Abcam	Ab124964
CGRP	1:2000	sheep	Enzo Life Sciences	BML-CA1137-0100
VAChT	1:1000	rabbit	Synaptic Systems	sysy139103
5-HT	1: 5000	rabbit	Cell Signalling	ABIN61783
Secondary Antibodies	**Dilution**	**Host species**	**Manufacturer**	**Catalog #**
Rabbit/Alexa 488	1: 1000	Donkey	ThermoFisher	A21206
Sheep/Alexa 568	1: 1000	Goat	ThermoFisher	A21099

5.5. HPLC

Quantification of 5-HT in the L5-S1 spinal cord was performed by HPLC, as previously described [24]. Stored fresh tissue at -80°C was immersed in ice-cold perchloric acid for 2h before analysis. Samples were filtered on Costar Spin-X microfilter tubes, and 50 µL of the eluate was injected into an HPLC. The lower limit for detection was 500 fmol.

5.6. Sirius Red Staining

Frozen urethral sections were left to dry at room temperature for 1h before staining with Picro-sirius red solution for 90 min. The sections were then washed 2 times with 0,5 % acidified water and dehydrated in three changes of 100 % ethanol and two benzene baths. Sections were mounted with Entellan definitive mounting medium.

5.7 Administration of pharmacological antagonists of 5-HT receptors (5-HT_2 and 5-HT_3)

To test whether pharmacological modulation of urethral 5-HT signalling pathways could result in ameliorated urinary function, Ritanserin (Cat. No. 1955. Tocris Bristol, UK), a potent, non-selective and long-lasting 5-HT_2 receptor antagonist, and Ondansetron hydrochloride (Cat. No.

2891. Tocris Bristol, UK), a selective 5-HT_3 receptor antagonist, were administered to additional groups of WT mice. Per the manufacturer's instructions, drugs were reconstituted in ethanol (Ritanserin) and distilled H_2O (Ondansetron Hydrochloride) and stock solutions were stored at -80°C. Female WT mice underwent spinal cord transection surgeries at the T8/T9 spinal level, as described above, and were divided into four experimental groups: 1) Ritanserin (RT) (1mg/Kg/day); 2) Ondansetron (ON) (1mg/Kg/day); 3) Ritanserin+Ondansetron (RT+ON) (1mg/Kg/day + 1mg/Kg/day); and 4) Vehicle (ethanol+H_2O in the same concentration as used for drugs dilutions). Aliquots of Ritanserin and Ondansetron's stock solutions were diluted in saline and delivered via intraperitoneal injection, starting on surgery day and spanning 4 weeks post-SCI. Drugs and dosages were chosen according to previous studies [24, 28]. Retained urine volumes were recorded every three days upon daily bladder compression in all groups, since SCI induction and throughout the protocol. After 4 weeks of treatment, all animals underwent cystometries as described in 5.3.

5.8 Data Analysis

Cystometrograms were analysed using LabScribe software (World Precision Instruments, Hertfordshire, UK). Animals suffering from bladder overflow induced by anaesthesia were excluded. For immunohistochemistry quantifications, three to five longitudinal urethral sections per animal were considered and quantified by densitometry in Fiji, as before [27]. The results are presented as an average of the values measured in the posterior (closer to the vagina) and anterior side of the urethral wall. Data was collected near the sphincter region of the proximal urethra. Fibrosis levels were quantified as the area of collagen red-stained fibres compared to the total tissue area. All measurements were performed on raw files. For exemplificative images, small adjustments in luminosity and/or contrast might have been made. Data was statistically analysed using one or two-way ANOVA followed by Tukey's multiple comparison post hoc test

using GraphPad Prism 9 software. Results are represented as mean ± SD, and $p<0.05$ was considered statistically significant.

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AUTHOR CONTRIBUTION

AF: Conceptualisation, methodology, formal analysis, original draft, writing, and editing

SSC, AA: Methodology

CR: Methodology, formal analysis, and editing

CDR: Conceptualisation, methodology, formal analysis, writing, and editing

All authors read and approved the final manuscript.

DECLARATION OF FUNDING AND/OR CONFLICTS OF INTERESTS/COMPETING INTERESTS

The authors declare they have no conflict of interest. No funds were received from industry, and there are no commercial or financial incentives associated with the publication of this work.

DATA AVAILABILITY STATEMENT

Data will be made available upon reasonable request.

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Final Considerations

In healthy individuals, switching between urine storage and voiding is controlled by a complex neuronal network that integrates afferent and efferent signals between the lower urinary tract (LUT) and supraspinal centres (Fowler et al., 2008). Lesions occurring at any point along the neuroaxis can lead to neurogenic detrusor overactivity (NDO), as is the case with spinal cord injuries (SCI). Animal models of SCI are commonly used to study NDO *in vivo*, where urinary impairment results from the abrupt cessation of communication between the LUT and supraspinal centres as a result of a spinal trauma (Fry et al., 2010; Doelman et al., 2023). After an initial period of spinal shock, the emergence of an automatic maladaptive micturition reflex results in NDO, often concurrent with detrusor-sphincter dyssynergia (DSD), ultimately leading to urinary incontinence and further urinary complications (de Groat and Yoshimura, 2010).

The impact of SCI-induced urinary dysfunction extends far beyond physical limitations, triggering severe psychological distress associated with the sudden deterioration of patients' quality of life (James et al., 2019). For this reason, regaining bladder control stands as an absolute priority for patients, sometimes even surpassing the desire to restore walking ability (Anderson, 2004). Unfortunately, available therapies for SCI-induced urinary incontinence remain merely symptomatic and are applied only when incontinence is already established, with no possibility of cure. These interventions are based on drugs that aim to prevent bladder hypercontractility by blocking detrusor muscle contraction, requiring intermittent catheterisation for urine removal. However, these drugs offer limited therapeutic efficacy that loses efficacy over time and are often accompanied by severe side effects that range from constipation to cognitive impairment. These adverse side effects compromise treatment adherence and prompt

patients to abandon treatments and further deteriorate their independence and dignity (Wyndaele, 2016; Sartori et al., 2024).

Our understanding of the pathophysiological mechanisms of SCI and its consequences has heavily relied on animal models of disease. Although emerging innovative non-animal techniques, such as organ-on-a-chip systems, computational models, and in vitro neural-bladder co-culture platforms, have been useful to understand some features of the pathology, they still cannot replicate the complex neuronal interactions between LUT organs and the central nervous system. SCI disrupts this communication at multiple levels, leading to diverse and dynamic patterns of dysfunction that can only be understood using a living organism. Part of this dissertation focused on investigating the use of animal models in NDO research, first by providing a systematic review of animal models used in NDO research (***Publication I***), which are mainly dependent on SCI models, followed by a comparative analysis of urinary outcomes induced by different SCI protocols also focusing on the urethra as a poorly studied LUT organ in this context (***Publication II***).

Traditionally, the bladder stood as the central organ in SCI-induced NDO emergence, and thus the primary target of pharmacological intervention. The urethra was regarded as a simple conduit for urine expulsion, and little attention was paid to its role in LUT sensory pathways or its eventual contribution to LUT dysfunction. However, advances in urological research have challenged this view, with new findings demonstrating not only the role of urethral afferent input in promoting efficient bladder emptying, but also in regulating continence mechanisms (Birder et al., 2014; Eggermont et al., 2019; Ferreira and Duarte Cruz, 2021), raising the question of whether NDO could also reflect altered urethral activity. Therefore, some of the work included in this

dissertation shifted the common focus of SCI-induced urinary dysfunction from the bladder to the urethra and investigated the consequences of SCI on this organ (***Publication III***), as well as its effects on bladder-urethral communication mediated by peripheral urethral serotonin (5-HT) (***Publication IV***).

The experimental work was performed using rodent animal models. Rats (*Rattus norvegicus*) were used in ***Publications II and III***, whereas mice (*Mus musculus*) were preferred in ***Publication IV***, as this species allowed the use of genetically modified animals crucial in our studies. Most animals underwent complete spinal cord transection (SCT) at the T8/T9 spinal level, an animal model used by our group to study SCI-associated urinary dysfunction for over twenty years (Cruz et al., 2006; Cruz et al., 2008; Frias et al., 2015; Coelho et al., 2016; Oliveira et al., 2019; Chambel et al., 2022). Despite acknowledging that complete SCT injuries are uncommon in human patients, the simplicity of the technique allows for the induction of homogeneous experimental groups with a reliable, consistent, and reproducible pattern of urinary impairment. Nonetheless, different types of injury, such as incomplete or contusion injuries, likely reflect distinct molecular mechanisms of disease, a matter discussed in ***Publication II***.

All experimental procedures were performed on female animals, as the female urethra is anatomically shorter and easier to manipulate than the male counterpart, thus facilitating manual bladder emptying and, if needed, catheterisation for urine removal. We are aware that this choice presents a limitation to the work, as most SCI cases occur amongst the male population (Raguindin et al., 2021). Besides, there is evidence of important differences between male and female micturition behaviours. These arise not only from differences in urethral anatomies but also from neurophysiological and hormonal factors, which can impact the pathophysiology of SCI and neuronal recovery

(Sipski et al., 2004), highlighting the need to use both sexes in SCI-associated urinary research in future studies.

1. **Basic research concerning NDO is mostly based on models of SCI: Implications of model type, species and gender choice**

The results included in ***Publications II, III and IV*** relied on rodent models of complete spinal cord transection, which resulted in consistent and predictive patterns of NDO, which were similar in these publications. NDO is the most frequent LUT disorder seen in neurological patients, with vast etiological origins. Nevertheless, NDO has similar clinical manifestations (Panicker et al., 2015), highlighting the value of standardised experimental models for elucidating pathophysiological mechanisms and evaluating potential therapeutic options. ***Publication I*** comprises a systematic review of currently used animal models in NDO research, specifically in central nervous system (CNS) pathologies. Animal models of suprasacral SCI stood as the favoured models to induce NDO, due to their wider lesion homogeneity and predictable functional outcomes. Experimental SCI is more commonly induced by complete spinal cord transection or hemisection, followed by spinal cord contusion. As most SCI occur from blunt trauma during a traumatic incident, when the research goal is to investigate injury pathophysiological processes or repair mechanisms, contusion models should be preferred as they closely reproduce human injuries. In urological research, however, it is also fundamental to attain a consistent and predictable phenotype of bladder dysfunction, which is easily achieved by using a highly reproducible model such as the complete spinal cord transection. Despite being technically simple and less costly, SCT injuries are associated with poor translational value when compared with contusion

injuries, as they are less frequent than contusions in human patients. Actually, several studies have suggested that the gravity of urinary impairment reflects the type and the severity of the injury (Mitsui et al., 2014; Breyer et al., 2017; Munoz, 2017), something we also observed in ***Publication II***, with probable implications for translating basic research data to the clinics. Of note, most experimental SCIs are induced at the thoracic level, contrasting with human SCI, where the cervical segments are most commonly affected (Lee et al., 2013). This occurs because cervical injuries are associated with respiratory compromise, which is complicated to control in the experimental setting. Therefore, they result in higher mortality rates and are, for this reason, avoided in animal models (Lane et al., 2008).

Publication I also highlight rodents as the preferred animal model to induce NDO. Rats are frequently chosen, due to their low maintenance costs, well-understood anatomy and physiology, as well as their larger size (Fry et al., 2010). Mice are less commonly used but are particularly important in studies requiring genetic editing. Even considering that mouse LUT morphology is more similar to that of humans, their biggest limitation is their small size, which limits their use in protocols requiring complex surgical procedures (Andersson et al., 2011). Larger animals, including rabbits or small primates, could provide a more accurate evaluation of outcomes, as they share increased morphological and disease pathophysiological similarity with human patients (Pradidarcheep et al., 2011). However, their maintenance costs and strict ethical regulations associated with their use preclude wider implementation. Females are predominantly used, as their LUT anatomy and absence of prostate facilitates bladder and urethral manipulation. However, these morphological differences, allied to other sex-specific mechanisms including distinct profiles of sexual hormones, are responsible

for gender dysmorphisms in micturition behaviours (Sipski et al., 2004), a matter that highlights the need to use both sexes in future SCI-related urological research.

The analysis of the studies included in ***Publication I*** also allowed a systematic review of the multidimensional molecular and cellular pathways involved in NDO emergence. Results were discussed in terms of alterations in LUT tissue and changes in the neuronal content in both peripheral and central areas of the nervous system. Unsurprisingly, more attention was paid to the bladder at the detriment of the urethra, which was rarely referred to in the included studies. This confirms the big knowledge gap about alterations in the urethra after SCI and whether or not these changes produce an influence on post-SCI urinary function, a matter addressed in studies included in this dissertation (***Publications III and IV***).

2. Different models of SCI induce distinct effects on the lower urinary tract

Publication I showed that spinal transection, complete or incomplete, and spinal cord contusion (SCC) are the most commonly used animal models in SCI research, but raised awareness about discrepancies in urinary impairment features between the two models. ***Publication II*** explored these differences in the female rat, providing an overview of the urinary consequences and features of neuronal rearrangement preceding SCI-induced urinary dysfunction. As expected, the progression of bladder dysfunction differed between SCI models. Contusion injuries resulted in a broader area of damage to the spinal cord, with notable injury in extended areas well beyond the injury site. This might delay tissue healing and contribute to SCI-related immune depression (Jeffries and Tom, 2021; Rodgers et al., 2022), resulting in longer spinal shock periods and increased urinary retention. Mildly contused animals presented less severe signs of urinary dysfunction,

likely due to the existence of larger areas of preserved tissue, suggesting a positive correlation between the severity of injury and the severity of urinary impairment as suggested by others (Pikov and Wrathall, 2001; Leung et al., 2007; Mitsui et al., 2014; Breyer et al., 2017). SCT animals presented fewer urinary complications, possibly due to their restricted area of damaged tissue in the spinal cord. Nevertheless, these differences were not reflected in urodynamics, as the frequency and amplitude of bladder contractions were similar between groups, raising the question of whether possible urodynamic differences were obscured by the use of urethane as anaesthetic for cystometries (Cheng et al., 1995; Yoshiyama et al., 2013).

Different types of SCI also led to distinct neuronal circuitry rearrangements in the LUT. Signs of sensory and cholinergic denervation were seen in the bladder and urethra of all injured rats, as indicated by downregulation of CGRP and VAChT expression. Noradrenergic fibres (TH^+) were only detected in the urethra, where post-SCI denervation was also observed, particularly in contusion models, although a tendency for TH loss was also seen in SCT animals. On the other hand, the opposite occurred at the lumbosacral spinal cord level. In this region, expression of $GAP43^+$, an established marker of axonal sprouting (Benowitz and Routtenberg, 1997), which co-localised with CGRP, was increased after injury, particularly in the mild contusion model, reflecting the current knowledge of SCI-induced NDO emergence (Zinck et al., 2007; de Groat and Yoshimura, 2010). However, without neuronal tracing, we were unable to assess whether these central projections of sensory neurons belong to afferents innervating the bladder and/or the urethra. Reflecting the disruption of the central parasympathetic projections after SCI (Roy and Green, 2019), expression of VAChT, a cholinergic marker, was decreased at the lumbosacral spinal cord, as in the LUT. Nonetheless, in contrast to

the bladder and urethra, the pattern of denervation was dependent on injury severity, as mildly contused animals did not undergo significant parasympathetic loss, unlike the more severe models. It is acknowledged that loss of cholinergic fibres in response to SCI is initiated by a dramatic decrease during spinal shock, which is partially recovered as NDO develops (Takahara et al., 2007). It is possible that mild contusion animals lost $VAChT^{+}$ innervation in an earlier stage but later were able to restore it. The tendency for decreased VAChT expression in severely contused animals, compared to SCT animals, supports this hypothesis. Finally, noradrenergic denervation was seen in all SCI animals, regardless of the injury model. This work provided important evidence about the influence of the model chosen to inflict injury, highlighting how variations in experimental design can affect results, particularly in the study of urinary dysfunction after SCI.

3. SCI induced distinct patterns of urinary dysfunction in rats and mice: Implications for translational research?

The timepoints of SCI progression used throughout this dissertation were chosen based on the hallmarks of post-SCI urinary dysfunction observed in the rat. One week post-injury represents the period of spinal shock, during which the bladder presents little or no contractility due to the abrupt cessation of supraspinal input (Bywater et al., 2018; Anderson et al., 2023). During this period, which lingers for approximately 2 weeks in rats, urine was removed by abdominal compression until automatic voiding was established. Four weeks post-injury represents the period in which NDO has already emerged and urinary incontinence is established (Anderson et al., 2023), with a

noteworthy increase in the frequency of bladder contractions alongside a decrease in amplitude, compared to uninjured rats (***Publications II and III***).

The same timepoints were chosen in experiments using mice. Unlike rats, signs of bladder contractility were already present 1 week post-injury (***Publication IV***). Nevertheless, mice required abdominal compression for urine removal throughout the entire protocol and never developed satisfactory automatic voiding. It is established that rat urinary voiding requires external urethral sphincter (EUS) bursting activity for urine expulsion (LaPallo et al., 2014; Langdale and Grill, 2016). Mice, on the other hand, require long periods of EUS relaxation for continuous urine flow. This feature probably renders mice more prone to DSD, as this relaxation period is rarely prolonged enough to achieve voiding, resulting in intermittent urine expulsion associated with slow reductions in intravesical pressure during periods of low EUS activity (Kadekawa et al., 2016). Together with others, our observations suggest that these distinct voiding patterns between mice and rats might be related to species-specific variances in urethral activity during voiding, possibly shaping distinct mechanisms of functional recovery following SCI. Unfortunately, we were not able to assess urethral activity in either SCI rats or mice, a matter that should be addressed in future studies.

It is important to note that mouse EUS firing during urine voiding closely mirrors that of humans, where voiding occurs during reduced EUS activity (Kadekawa et al., 2016; Wada et al., 2022). Even considering that this feature predisposes mice to DSD and possibly worsens urinary retention and possible LUT infections, as observed in ***Publication IV***, allied to their vulnerability to post-surgical complications, mouse models stand as a powerful translational model for advancing our understanding of urethral pathology and recovery after SCI.

The methods used here for urodynamic recording also warrant consideration for future studies to ensure translational value. Bladder reflex activity was assessed by cystometries under urethane anaesthesia (***Publications II, III and IV***), which, even if minimally affecting bladder contractility, disrupts EUS activity and sphincter coordination (Yoshiyama et al., 2013). As urethral function was a matter of central importance in this dissertation, the evaluation of bladder contractility in an awake state would have been preferred. Unlike anaesthetised cystometry, which is restricted to a single recording, awake cystometry may allow longitudinal repeated LUT monitoring, as well as simultaneous recording of bladder and urethral function. This would be important for the detection and characterisation of different degrees of DSD (Koyanagi et al., 1982; Weld et al., 2000). Nonetheless, awake urodynamic recording requires sophisticated and costly equipment, as well as complex surgical procedures for implantation and maintenance of catheters and/or electrodes, particularly challenging in mice, which were not feasible within the constraints of available resources.

4. SCI-induced urinary impairment coursed with significant alterations in the urethral tissue

The urethra plays a critical role in the neuronal control of micturition, influencing not only the mechanisms underlying urinary incontinence, but also has a renowned role during voiding. Given the impact of SCI on urinary function, it comes as no surprise that the post-SCI urinary impairment was accompanied by profound alterations in the urethra (***Publication III and IV***). At the urethral mucosa, SCI induced cellular desquamation and histological disorganisation in the female rat during spinal shock, partially resolved when NDO was already established 4 weeks post-injury. At this time point, epithelial thickness

was increased, reflecting a higher cellular turnover, which has been previously described in the bladder of both rats and mice in response to SCI-induced stress on the bladder epithelial lining (Apodaca et al., 2003; Kullmann et al., 2017) (***Publication III***). Evidence of epithelial reorganisation was also seen in SCI mice, where 5-HT^{+} epithelial paraneuronal cells were increased in chronic SCI (***Publication IV***).

SCI induced atrophy of the smooth muscle fibres of the internal urethral sphincter (IUS) of both rats (***Publication III***) and mice (***Publication IV***). This contrasts with the SCI bladder, where smooth fibres of the detrusor muscle undergo hypertrophy and fibrosis to deal with the enlarged bladder capacity and continually elevated intravesical pressures (Deveaud et al., 1998; Toosi et al., 2008; Johnston et al., 2012). The IUS may behave differently, as the urethra does not undergo such elevated pressures and distension as the bladder after SCI. Moreover, sympathetic denervation co

In SCI rats, alterations in the external urethral sphincter (EUS) were also observed, including increased fibrosis (***Publication III***). However, the physiology of the mouse EUS remained unchanged by SCI (***Publication IV***), possibly due to the distinct EUS activity in both species, as discussed above. Urine voiding in the rat courses with EUS phasic contraction, which, after SCI, becomes abnormal, presenting either uncoordinated or tonic activity due to the emergence of DSD (Kruse et al., 1993; Abud et al., 2015; LaPallo et al., 2017). This induces a mechanical overload, as well as ischemia on muscle fibres, probably leading to muscle damage and fibrotic remodelling, as seen in other muscular cell types after SCI (Garg et al., 2015). As voiding in the mouse relies on EUS relaxation, the fibrotic response in urethral striated cells after SCI might be attenuated, at least in wild-type mice (***Publication IV***).

Urethral morphological alterations were accompanied by rearrangements of nerve fibres in its wall. In the rat, there was significant denervation after SCI, compromising both the innervation of the mucosal and muscular layers during the spinal shock stage (***Publications II and III***). This denervation affected sensory peptidergic nerve fibres ($CGRP^+$) and noradrenergic fibres (TH^+), reflecting the interruption of both afferent and efferent pathways. The impact of SCI on urethral cholinergic fibres ($VAChT^+$) was unclear. In ***Publication III***, no effect of SCI on urethral cholinergic neurons was observed. On the other hand, in ***Publication II,*** $VAChT^+$ fibres were significantly reduced after SCI. The latter appears to be more in line with observations in the bladder, where SCI induces cholinergic loss in the detrusor muscle (Takahara et al., 2007) as a result of damage or shrinkage of cholinergic neurons in relevant central motor pools (Hou and Rabchevsky, 2014). It is important to note that cholinergic signalling in the urethra depends not only on acetylcholine (Ach), which is mostly responsible for smooth muscle contraction, but also on nitric oxide (NO), an inhibitory messenger that induces smooth muscle relaxation (WC. de Groat, 2001). Both signals interact functionally to assure proper urethral sphincter function in intact conditions but are dysregulated in the bladder after SCI (Artim et al., 2011; Kanai et al., 2023). In the urethra, though, the effects of spinal lesions on NO signalling are unknown, and their clarification could help to understand alterations in cholinergic signalling after SCI.

In mice, post-SCI urethral innervation showed an intriguingly opposite pattern to that observed in rats. Instead of neuronal fibre loss, an increase in the expression of sensory and cholinergic neuronal markers was seen, particularly in the IUS region, suggesting urethral fibre upregulation following SCI (***Publication IV***). Again, distinct SCI progression mechanisms between mice and rats might contribute to these differences, as the rapid

re-emergence of bladder contractility observed in mice (***Publication IV***) could result from accelerated neuronal sprouting. This is a matter worth pursuing in future studies, by double-labelling sensory and cholinergic fibres with a marker of axonal sprouting, such as GAP-43, in the SCI mice urethra. Understanding these interspecies differences and relating them to post-SCI urethral dysfunction is essential for a deeper knowledge of the big picture beyond SCI-induced urinary dysfunction. Therefore, it became important to investigate how specific signalling pathways contribute to urethral dysfunction after SCI, as is the case with the urethral serotonergic system, which has gained attention for its role in modulating urethral sensory pathways and regulation of bladder function.

5. Post-SCI urinary impairment reflects upregulation of urethral serotonergic signalling

The existence of an urethro-vesical crosstalk regulating bladder function has been extensively documented in humans and experimental models (Birder et al., 2014; Coelho et al., 2018; Kullmann et al., 2018; Eggermont et al., 2019; Ferreira and Duarte Cruz, 2021). One of the players in this crosstalk is peripheral 5-HT, locally released by urethral paraneurons as the urine flows during voiding, enhancing urethral afferents' excitability, and culminating in enhanced voiding efficiency, by mechanisms that are still not fully understood (Kullmann et al., 2018) (***Publication III***). The effects of SCI on this mechanism were studied in ***Publication IV***, with a genetic mouse model. These animals lack peripheral tryptophan hydroxylase 1, the rate-limiting enzyme necessary for peripheral synthesis of serotonin, and are, thus, *tph1* $^{-/-}$, allowing for a deeper understanding of the role of peripheral 5-HT in post-SCI LUT function. Central production of serotonin is not

affected, as we found no differences in 5-HT levels at the lumbosacral spinal cord in spinal-intact and SCI animals, irrespective of their genotype.

Wild-type mice presented, as expected, disrupted bladder function in response to SCI. Signs of bladder hyperactivity were accompanied by increased expression of sensory ($CGRP^+$) and cholinergic markers ($VAChT^+$) in the IUS region, which coursed with increased expression of 5-HT^+ paraneurons in the urethral epithelium. The number of 5-HT^+ cells was the highest at 4 weeks post-SCI, when NDO was already established. These cells were located in deep layers of the urethral epithelium, in the vicinity of nerve fibres. It is possible that, upon stimulation, these cells release 5-HT that binds to these nerve fibres, which may express the serotonin receptors 5-HT2 and 5-HT_3. Although we were not technically able to detect their presence, other studies have demonstrated their functional relevance in the LUT, and the binding of 5-HT to their receptors has been linked to afferent hyperexcitability and bladder overactivity (Coelho et al., 2018; Kullmann et al., 2018). It should be noted that other studies have demonstrated the involvement of the 5-HT_2 and 5-HT_3 receptors in non-neurogenic bladder hyperactivity, such as cystitis/painful bladder syndrome, where elevated 5-HT levels in the LUT have similarly been demonstrated (Chuang et al., 2016; Ikeda et al., 2018; Chen et al., 2024).

In contrast, and as expected, it was not possible to identify 5-HT^+ cells in the urethral epithelium of *tph1* $^{-/-}$ mice. Still, *tph1* $^{-/-}$ mice developed some degree of bladder dysfunction, but less severe than that observed in wild-type littermates. Interestingly, in transgenic mice, urinary impairment was not accompanied by upregulation of sensory and cholinergic markers, as in WT mice, suggesting that serotonin might have a trophic effect on CGRP and VAChT expression. In the absence of peripheral 5-HT, bladder dysfunction likely results from mechanisms independent of serotonin. We also observed

IUS atrophy at 4 weeks post-SCI in both genotypes, but fibrosis was only in the EUS of *tph1* $^{-/-}$ mice. Serotonin is known to have a trophic effect on smooth muscle fibres (Wouters et al., 2007; Ambrogi et al., 2025), as well as a pro-fibrotic role in tissue remodelling (Mann and Oakley, 2013; Bi et al., 2017; Zhang et al., 2018; Pang et al., 2021), contradicting our findings. Nevertheless, these are effects reported under non-injured conditions, different from the highly noxious and pro-inflammatory environment known to occur following SCI (Ma et al., 2023; DiSabato et al., 2024). Moreover, as peripheral 5-HT regulates immune responses (Hodo et al., 2020), it is possible that in the context of SCI, the absence of 5-HT could trigger alternative inflammatory mechanisms that lead to enhanced IUS atrophy and EUS fibrosis.

In intact conditions, peripheral 5-HT appeared to have a minor role in LUT function, as no significant differences in urodynamics were detected between WT and *tph1*$^{-/-}$ mice. This finding could be explained by comparing with the role of peripheral 5-HT in the gastrointestinal (GI) tract, where peripheral 5-HT accounts 98% of the body's total 5-HT (Berger et al., 2009) but has a limited role in normal GI tract function, that, instead, mostly depends on centrally derived 5-HT (Terry and Margolis, 2017). Under inflammatory conditions, however, peripheral 5-HT assumes a more prominent role, initiating a cascade of inflammatory responses that amplify gastrointestinal activity and catalyse the emergence of inflammatory symptoms (Ghia et al., 2009; Margolis et al., 2014). A similar response may take place in the urethra after SCI, where the drop in central 5-HT availability seen at the lumbosacral spinal cord level (***Publication IV***) might prompt a compensatory increase in peripheral 5-HT synthesis. This could hyperactivate urethral afferents and enhance bladder contractility, further contributing to inflammation and urinary dysfunction. To test this hypothesis, it would be interesting to

restore central 5-HT levels in SCI mice, for instance, with intrathecal delivery of 5-HT or its precursor 5-Hydroxytryptophan (5-HTP) to the lumbosacral spinal cord, combined with a peripheral decarboxylase inhibitor such as carbidopa. This could also be achieved by viral delivery of *tph2* to induce lumbosacral expression of 5-HT. In any case, peripheral and central 5-HT levels should be measured as well as the effects on bladder function.

6. Modulation of peripheral 5-HT signalling by the blockade of serotonergic receptors failed to ameliorate NDO symptoms

As bladder function of mice lacking peripheral 5-HT appeared to be less compromised than that of their WT counterparts, we hypothesised whether modulating urethral 5-HT receptors in injured WT animals could be a viable therapeutic strategy to prevent NDO emergence after SCI. The daily blockade of 5-HT_2 receptors with Ritaserin during 28 days induced increased amplitude of bladder contractions, possibly reflecting increased ability to store urine and improved voiding in these animals. Nevertheless, these animals presented suggestive signs of severe urinary infections that sometimes required anaesthetised catheterisation for urine removal. On the other hand, the blockade of 5-HT3 receptors with Ondansetron did not result in altered bladder function but did induce increased incidence of urinary infections when compared to vehicle-treated mice. However, they tended to be milder than the ones observed in ritanserin-treated mice. The combined treatment of the two drugs did not altered bladder function, yet the incidence of urinary infections remained high, suggesting that Ondansetron counteracted the excitatory effects of Ritanserin on bladder contractility (***Publication IV***).

The lack of significant ameliorative effects of Ondansetron might be associated with wrong drug dosing rather than a lack of pharmacological effects. Drug dosages were

chosen based on prior studies in intact animals (Kullmann et al., 2018; Sattayachiti et al., 2022), but SCI might influence drug absorption and metabolism, leading to reduced efficacy. Performing a preliminary dose-response study identifying the optimal response dose before full-scale experimentation would have been the most appropriate approach. It is also important to note that because treatments were delivered systemically, 5-HT receptor modulation was not restricted to the urethra, which could have also led to blockade in other locations such as the bladder, where these receptors are also expressed (Konthapakdee et al., 2019; Chen et al., 2024). In future studies, a targeted blockade restricted to the urethra might produce distinct functional outcomes and offer clearer insights into the specific role of urethral 5-HT signalling in LUT function.

Conclusions

The present dissertation focused on investigating the mechanisms underlying central injuries, particularly SCIs, in the LUT function of rodents, with a particular focus on the urethra. The main findings of these studies were:

1. SCI models are the preferred method to induce NDO *in vivo*, with contusion and complete transection models being the most chosen methods.
2. Less severe types of SCI are associated with milder urinary dysfunction symptoms, reflecting distinct rates of afferent and efferent neuronal rearrangement at the lumbosacral spinal cord level.
3. SCI, resulting from complete spinal cord transection, induces morphological rearrangement in urethral tissues, including epithelial disorganisation, smooth muscle atrophy and striated muscle fibrosis.
4. Post-SCI urethral morphological rearrangement is accompanied by significant neuronal reorganisation, which is distinct in mice and rats.
5. SCI induces upregulation of urethral-produced serotonin, as evidenced by increased serotonergic paraneurons lining the urethra, which correlated with the emergence of post-SCI urinary impairment.
6. Peripheral 5-HT influences post-SCI urethral changes in smooth muscle integrity and neuronal rearrangement, which possibly mediate distinct mechanisms of NDO emergence.

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